PYROPHOSPHATE ANALOGUES AS ANTIVIRAL AGENTS

by

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ABBREVIATIONS

Acyclovir	9-(2-Hydroxyethoxymethyl)guanine
AI-ddUrd	5'-Amino-5-iodo-2',5'-dideoxyuridine
Amantadine	1-Adamantanamine
(d)AMP-PAA	(deoxy)Adenosine 5'-phosphorophosphono-acetate
AMP-PCP	Adenosine 5'-[β,γ-methylene]triphosphate
AMP-PC$(Cl)_2$P	Adenosine 5'-[β,γ-dichloromethylene]-triphosphate
Ara-A	9-β-D-Arabinofuranosyladenine
Ara-T	1-β-D-Arabinofuranosylthymine
BV-dUrd	E-5-(2-Bromovinyl)-2'-deoxyuridine
CI	Chemical ionisation
CMV	Cytomegalovirus
c.p.e.	Cytopathic effect
DMEM	Dulbecco's modification of Eagle's medium
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
EI	Electron impact
FANA	2-Deoxy-2,3-dehydro-N-trifluoro-acetylneuraminic acid
FIAra-C	2'-Fluoro-5-iodo-1-β-D-arabino-furanosylcytosine
GMEM	Glasgow modification of Eagle's medium
H	Haemagglutinin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HSV, -1, -2	Herpes simplex virus, type 1, type 2
5-Iodo-dCyd	5-Iodo-2'-deoxycytidine
5-Iodo-dUrd (Idoxuridine)	5-Iodo-2'-deoxyuridine
M	Matrix
MalNet	N-Ethylmaleimide
m.o.i.	Multiplicity of infection
N	Neuraminidase
NCS	Newborn calf serum
NEAA	Non-essential amino acids
(d)NMP-PAA	(deoxy)Nucleoside 5'-monophosphate covalently linked to phosphono-acetate by a phosphodiester bond
NP	Nucleoprotein
p^r	Phosphonoacetate resistant
p^s	Phosphonoacetate sensitive
PAA	Phosphonoacetic acid
PBS	Phosphate buffered saline
PFA	Phosphonoformic acid
pfu	Plaque forming units
$pK_{d'}$	Dissociation constant for complex formed between zinc ions and a pyrophosphate analogue at pH 8.0
$pp(Ap^2)_2A$	5'-Triphosphate of adenyl(2'-5') adenyl(2'-5')adenosine
Ribavirin	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine
Rimantadine	α-Methyl-1-adamantane-methylamine
TK	Thymidine(deoxycytidine)kinase
VAP	Virus attachment protein
VZV	Varicella-zoster virus

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DECLARATION

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick and at the Department of Applied Biology, Roche Products Ltd., Welwyn Garden City, between October, 1980 and September, 1983, and has not been submitted previously for a degree at any institution.

***

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***

PUBLICATIONS

Parts of the research described in this thesis have appeared in the scientific literature as follows:

1. Ammonia Chemical Ionisation Mass Spectra of Esters and Amides of Oxyacids of Phosphorus
Cload, P. A. and Hutchinson, D. W. (1983)
Organ. Mass Spectrom., 18, 57-59

2. Organophosphorus Compounds as Antiviral Agents
Hutchinson, D. W., Cload, P. A. and Haugh, M. C. (1983) *Phosphorus and Sulfur*, 14, 285-293

3. The Inhibition of the RNA Polymerase Activity of Influenza Virus A By Pyrophosphate Analogues
Cload, P. A. and Hutchinson, D. W. (1983)
Nucl. Acid Res., 11, 5621-5628

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ABSTRACT

The work described in this thesis was aimed at distinguishing between two possible mechanisms for the antiviral mode of action of pyrophosphate analogues. Either, (a) they are converted into analogues of nucleoside triphosphates which inhibit the viral polymerase, or (b) they interact directly with the polymerase possibly by coordinating with an essential metal ion.

Several pyrophosphate analogues have been synthesised and screened for activity against herpesvirus DNA polymerase, influenza RNA polymerase and calf thymus DNA polymerase α. Characterisation of a number of these compounds was accomplished by chemical ionisation mass spectrometry. A number of reagent gases were tested and ammonia was found to be the most satisfactory.

The work carried out in this thesis suggests that the pyrophosphate analogues do not inhibit the viral polymerases by first being incorporated into the β-γ positions of nucleoside triphosphates. The proposed nucleoside triphosphate analogues have been synthesised and these are neither substrates for, nor inhibitors of the enzymes. Furthermore, when [2-^{3}H]-phosphonoacetate was incubated with the standard polymerase assay mixtures, all the radioactivity recovered from the assays was in the form of starting material, none could be detected in the form of nucleoside triphosphate analogues.

However, it appears that the pyrophosphate analogues complex with an essential metal ion of influenza RNA polymerase. Dissociation constants ($K_{d'}$) for complexes formed between zinc ions and pyrophosphate analogues at pH 8.0 have been determined by gel filtration and there is a correlation between the $K_{d'}$ of an analogue and its effectiveness as an inhibitor of influenza RNA polymerase.

CHAPTER 1

GENERAL BACKGROUND

1.0 THE DISCOVERY OF VIRUSES

By the 1880's the pioneering work of Pasteur, Koch and others had proved conclusively the microbial-aetiology of a number of disease states. However, there were a considerable number of infectious diseases that could not be attributed to bacteria or protozoa. It was originally assumed that these pathogens were extremely small bacteria which could not be cultured because of a lack of knowledge of their nutritional requirements.

In 1892, Ivanovsky‡ performed the classical experiment on the sap of tobacco leaves infected with mosaic disease to show the presence of infective bacteria. This involved passing the sap through a filter which had previously been shown capable of excluding the smallest of bacteria. The filtered material remained infectious but Ivanovsky refused to accept his results concluding that some contamination had taken place. In 1898, Beijerinck‡ repeated Ivanovsky's experiments and unlike his predecessor recognised their importance. To describe this unprecedentedly small pathogen Beijerinck coined the phrase "contagium vivum fluidum" (contagious living fluid) or "virus".

In the same year Loeffler and Frosch‡ demonstrated that cattle foot and mouth disease could be transmitted

‡ Dyson (1974)

by a filtrate. They extended this work by proving conclusively that no toxin was involved and speculated that many other diseases might be caused by these very small reproducing agents. During the early part of this century diseases such as mumps, chicken pox, rabies, yellow fever and small pox were shown to be caused by viruses (Table 1.0), and viruses have since been found to be ubiquitous parasites of living organisms.

1.1 MEDICAL EFFORTS TO CONTROL VIRAL INFECTIONS

Bacteria are totally independent entities containing many enzymes and substrates which are unique to the prokaryotic cell and afford useful targets for anti-bacterial drugs. The success of the general screening approach in the discovery of anti-bacterials is on the whole a reflection of the large number of processes which are found only in the bacterial cell. Unlike bacteria, which can multiply in tissues in an extracellular position, viruses are obligate intracellular parasites and can only multiply within cells which provide the synthetic and energy yielding processes which they lack.

The intimate intracellular relationship between virus and host led to the belief that viral diseases could not be treated with chemical agents since any effect on viral multiplication would necessitate a toxic effect on host processes. Hence, early efforts to combat viral infections were largely directed to preventing the individual from contracting the disease through personal

Table 1.O Important[a] Viruses of Vertebrates

Nucleic Acid Type	Family	Genus	Virus	Type of Infection Induced
DNA	Adenoviridae	*Mastadenovirus*	Numerous adeno types	Respiratory and ophthalmic
DNA	Herpetoviridae	*Herpesvirus*	Herpes types 1 and 2	Ophthalmic, central nervous system, genital, cutaneous, oral, upper respiratory
			Cytomegalo	Generalized of infant, respiratory, glandular
			Varicella (herpes zoster)	Nervous, cutaneous
			Pseudorabies	Central nervous system of livestock
			Infectious rhinotracheitis	Respiratory of livestock, fowl
DNA	Poxviridae	*Orthopoxvirus*	Variola (smallpox)	Generalized
			Vaccinia	Cutaneous
DNA	Papovaviridae	*Polyomavirus*	Polyoma, SV40	Tumors of rodents
RNA	Orthomyxoviridae	*Influenzavirus*	Influenza	Respiratory
RNA	Paramyxoviridae	*Paramyxovirus*	Parainfluenza	Respiratory
			Mumps	Glandular
			Newcastle disease	Respiratory and nervous of fowl
		Morbillivirus	Measles	Generalized
			Canine distemper	Generalized in dogs
		Pneumovirus	Respiratory syncytial	Respiratory
RNA	Picornaviridae	*Enterovirus*	Polio	Nervous
			Coxsackie	Respiratory, nervous, cardiovascular
			Echo	Nervous, intestinal
		Rhinovirus	Numerous rhino types	Respiratory
			Foot and mouth disease	Generalized of livestock
		Calicivirus	Feline calici	Respiratory of cats
			Vesicular exanthema	Generalized of swine
RNA	Rhabdoviridae	*Vesiculovirus*	Vesicular stomatitis	Generalized of livestock
		Lyssavirus	Rabies	Nervous
RNA	Coronaviridae	*Coronavirus*	Corona	Respiratory
			Infectious bronchitis	Respiratory of fowl
RNA	Togaviridae	*Alphavirus*	Eastern, Western, Venezuelan equine encephalomyelitis	Nervous, respiratory
			Semliki forest	Nervous
		Flavivirus	Yellow Fever	Nervous
			Numerous encephalitides, including Japanese B, St. Louis, Russian spring-summer	Nervous
			Dengue	Joints, cutaneous
		Rubivirus	Rubella	Skin, generalized, nervous
RNA	Bunyaviridae	*Bunyavirus*	Numerous encephalitides, including Bunyamwera and California	Nervous
RNA	Arenaviridae	*Arenavirus*	Lymphocytic choriomeningitis	Nervous
			Lassa	Generalized
RNA	Retroviridae	Types B and C *Oncovirus*	Numerous leukemias, including Friend, Gross, Moloney, Rauscher	Oncogenic of lower animals
			Rous sarcoma	Oncogenic of fowl

[a] Based on disease incidence or severity or on previous use in antiviral testing.

hygiene and immunisation and to the prevention of the spread of the disease through the community by the application of principles learned in the generalised study of epidemiology and public health.

1.1.1 Active Stimulation of the Immune Response

Prophylaxis by immunisation is an excellent way of preventing infectious disease and has been used in the control of many viral diseases using inactivated virus or live non-pathogenic virus and indeed the use of vaccinia virus immunisation has resulted in the eradication of smallpox. However, despite the undeniable success of vaccination in the fields of animal and human health not all virus diseases can be controlled in this manner. The reasons for this are many and varied; some are purely technical such as the purification in sufficient quantities of particular viral antigens, and may be overcome in due course but it is difficult to envisage how other, more fundamental problems may be overcome.

With some vaccines there is a small but not insignificant risk of complications such as the encephalitis which can follow smallpox immunisation. With live vaccines there is also the fear that the viral nucleic acid might be incorporated into the host genome or that the attenuated virus might revert to a pathogenic form.

Generally speaking, the risk of complications

is balanced against the probability of contracting the disease before any decision is taken by the health authorities to embark on a campaign of mass vaccination. However, the reluctance of some parents to allow their children to be vaccinated against a number of crippling and potentially fatal diseases has highlighted the public's fear of the adverse effects of some vaccines.

Infections of the upper respiratory tract are amongst the most common ailments of man (e.g. the common cold, influenza) yet the chances of producing adequate vaccines for such infections seem remote because of the large number of antigenic variants which are involved.

It is important to remember that immunisation is essentially a preventative measure; once a disease has been contracted vaccination has little hope of controlling its course in an individual unless, as in the case of rabies, the virus has a long incubation period. There is also a not insignificant percentage of the population who are immunocompromised and who cannot rely upon their immune systems to protect them from invading pathogens. In such individuals even a normally very mild infection such as herpes labialis (cold sores) can prove fatal (Nahmias and Roizman, 1973).

When one considers the problems associated with the production and administration of vaccines, their purely preventative nature and the incidence of immunocompromised patients it becomes evident that there is a real need for antiviral compounds which can

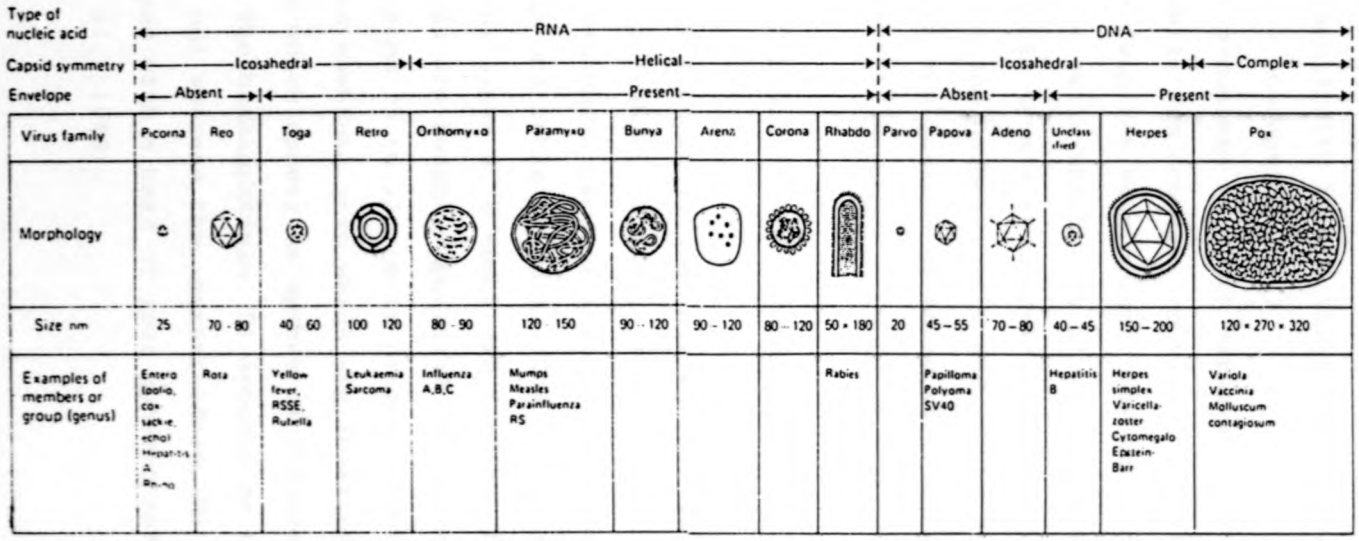
be used both prophylactically and therapeutically.

1.1.2 Introduction to Chemotherapy

The problem of viral chemotherapy has undergone many positive changes in the past two decades. The conceptual position that no specific antiviral compounds would ever be found because of the intimate association between virus and host cell has been shattered by the discovery, through random screening techniques, of a considerable number of antiviral agents. More importantly, advances in molecular virology have provided a detailed knowledge of many virus-specific processes and enzymes. Such information is clearly necessary if we are to develop, by rational means, specific antiviral drugs which have no effect upon the host cell.

1.2 VIRUS STRUCTURE AND REPLICATION

All known viruses contain either DNA or RNA, never both. The viral genome, which can be either single or double stranded, is enclosed and protected by a protein coat (capsid), which is made up of a number of identical subunits (capsomeres), often arranged in helical or cubical (or quasi-cubical) symmetry (Fig. 1.2). The protein coat is responsible for the majority of immunological responses that viruses induce in the host. The protein coat may itself be enclosed within an outer lipid membrane, which may



Virus family	Picorna	Reo	Toga	Retro	Orthomyxo	Paramyxo	Bunya	Arena	Corona	Rhabdo	Parvo	Papova	Adeno	Unclassified	Herpes	Pox
Morphology																
Size nm	25	70 - 80	40 60	100 120	80 - 90	120 150	90 - 120	90 - 120	80 - 120	50 × 180	20	45 – 55	70 – 80	40 – 45	150 – 200	120 × 270 × 320
Examples of members or group (genus)	Entero (polio, coxsackie, echo) Hepatitis A Rhino	Rota	Yellow fever, RSSE, Rubella	Leukaemia Sarcoma	Influenza A,B,C	Mumps Measles Parainfluenza RS				Rabies		Papilloma Polyoma SV40		Hepatitis B	Herpes simplex Varicella-zoster Cytomegalo Epstein-Barr	Variola Vaccinia Molluscum contagiosum

Fig. 1.2. Summary of structural properties of different RNA and DNA viruses

Lycke and Norrby (1983)

contain viral proteins. Certain virus-associated proteins act to lend specificity to the virus for the purpose of absorption to a susceptible cell.

The processes by which viruses replicate within susceptible cells are complex and vary from virus type to virus type from the very moment they encounter the exterior of the cells which they invade. The work described in this thesis is largely concerned with influenza virus and herpesviruses and so the structure, composition and replication of these viruses will now be discussed.

1.2.1 Influenza Virus: Structure and Composition

The influenza viruses are members of the myxovirus group (Table 1.2.1.i). They form a large group of viruses which produce acute respiratory disease in man and other animals. The structure of influenza virus types A, B and C are similar (Fig. 1.2.1). Virus particles are roughly spherical or filamentous in shape and some 75 to 120 nm in diameter bounded by an outer lipid membrane into which are inserted two glycoproteins which appear as spikes of different morphology. The haemagglutinin (H) appears as three-sided rods (14 x 4 nm) whereas the neuraminidase (N) is muchroom shaped with a head of four spheres and a tail (Varghese *et al.*, 1983).

Myxovirus	Source	Comments
Ortho-	Influenza Type A of: Man, swine, horse, duck, fowl	Share type specific nucleoprotein antigen. They all possess neuraminidase
	Influenza Type B	Distinctive nucleoprotein antigen. Recovered from man. Type B possesses neuraminidase, type C a R.D.E.
	Influenza Type C	
Para-	Mumps Newcastle Disease Influenza 1, 2, 3 and 4 Simian 5	All possess neuraminidase
	Measles, Distemper, Rinderpest	Do not possess neuraminidase

Table 1.2.1.i The Sub-Divisions of Myxovirus

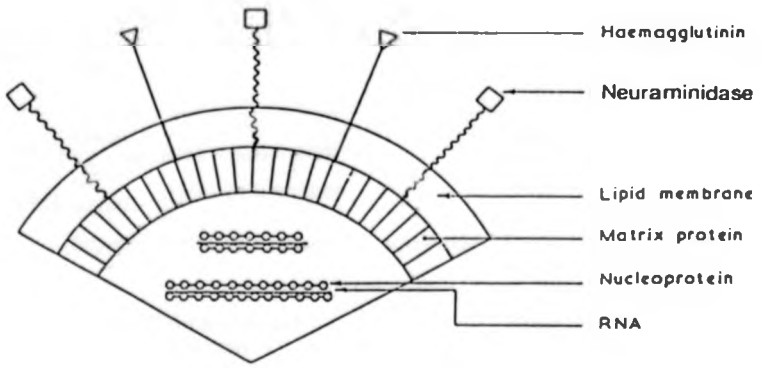


Fig. 1.2.1 Schematic diagram of influenza A virus (Laver and Valentine, 1969; Wrigley *et al.*, 1973).

Internally the virus particle contains five non-glycosylated proteins (Table 1.2.1.ii). The two major proteins are the nucleoprotein (NP) which is closely associated with the RNA genome and the matrix (M) protein which forms a shell underlying the lipid bilayer. Within the matrix shell and associated in some way with the genome are three high molecular weight proteins, designated P proteins, which form an RNA dependent RNA polymerase. The influenza virus type (A, B and C) is defined by the antigenicity of the ribonucleoprotein. Antibodies raised against the ribonucleoproteins of all type A viruses cross-react whereas no cross-reaction is observed with types B and C. Variation in the antigenicity of the H and N proteins is the basis of division of influenza type A viruses

Designation	Molecular Weight	Function	Approximate no. of molecules per virus particle	Assignment to structural and antigenic components of virus
PA PB_1 PB_2	range 81,000 to 94,000	RNA-dependent RNA-polymerase	50	Minor, internal non-glycosylated proteins. Unknown antigenic specificity.
N	60,000	Neuraminidase	100–200	Surface glycoprotein. Morphologically knob : fibre. Antigenically variable, subtype specificity
NP	53,000	Nucleocapsid sub-unit	1000	Internal, non-glycosylated protein associated with RNA to form helical nucleocapsid, type-specific, antigenically stable
HA1 HA2	*58,000 28,000	Haemagglutinin components	1000	Surface glycoproteins. Morphologically rods with triangular cross-section. Heavy (HA1) and light (HA2) polypeptide chains linked by disulphide bonds. Antigenically variable, subtype specificity
MP	25,000	Major, matrix or membrane protein	3000	Major internal non-glycosylated protein. Antigenically stable, type-specific. Associated with inner surface of lipid layer of envelope
NS1 NS2	23,000 11,000	Unknown "	— —	Non-structural virus-coded proteins. Synthesized in cytoplasm and migrate to nucleus of infected cells

* Haemagglutinin is synthesized as a single polypeptide of molecular weight 80,000 which is cleaved to HA1 and HA2 during virus maturation.
The values stated were determined for A Hong Kong 68 (H_3N_2) virus, X31 strain (Skehel and Schild, 1971).

Table 1.2.1.ii The Polypeptides of Influenza Virus
Stuart-Harris and Schild, (1976)

into sub-types (WHO memorandum, 1980; Hinshaw *et al.*, 1982).

The genetic material of influenza virus is single stranded, negative sense RNA which is segmented into 8 unique fragments each of which acts as a monocistronic message for a virus polypeptide (McGeoch *et al.*, 1976)‡. The segmented nature of the virus genome is responsible

‡ Genes 7 and 8 code for spliced mRNA's (McCauley and Mahy., 1983)

for the high frequency of genetic reassortment between influenza viruses which enables 'hybrid' viruses to be produced and may explain the appearance of new sub-types which occasionally cause pandemics (Webster, 1972; Webster *et al.*, 1982) since it can result in the appearance on the virus surface of new H and N glycoproteins to which populations have no immunity (Antigenic shift).

1.2.2 Stages of Influenza Virus Replication

The initial event in the infectious process is the absorption of the virus to the cell surface which is dependent upon two complementary structures; the receptor on the cell membrane and a region near the tip of the virus haemagglutinin. Synthetic oligopeptides which resemble the N-terminus of the haemagglutinin HA_2 sub-unit have been found to specifically inhibit the infectivity of influenza virus (Richardson *et al.*, 1980). These synthetic oligopeptides may interfere with the binding of the haemagglutinin to specific cell membrane receptor sites and so prevent the absorption of virus to the cell. The other virus glycoprotein, neuraminidase, does not appear to play a role in the initial interaction of virus and cell (Bucher and Palese, 1975).

The next step in the replication cycle is the penetration of the virus into the cell and there is considerable debate as to the precise mechanism by which this is achieved. There is some evidence to suggest that the virus particle enters by viropexis

(endocytosis) (Dourmashkin and Tyrrell, 1974). However, Morgan and Rowe (1968) and Waterfield *et al.* (1979) have proposed an alternative mechanism in which the virus and cell membranes fuse, thus allowing the release of virus 'core' material into the cell. Having gained entry to the cell the virus is then broken down into separated protein molecules and genetic material, a process known as uncoating. Again, controversy exists as to the actual mechanism by which this is achieved but it appears that cellular enzymes are involved. Two drugs, 1-adamantanamine (Amantadine) and α-methyl-1-adamantane-methylamine (Rimantadine) (Fig. 1.2.2.i), which are used clinically to treat influenza virus infections, seem

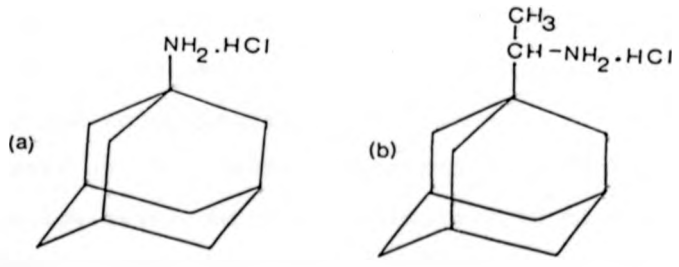


Fig. 1.2.2.i Amantadine hydrochloride (a) and Rimantadine hydrochloride (b).

to exert their antiviral effect at the penetration and/or uncoating stage(s) (Hossman *et al.*, 1965; Kato and Eggers, 1969; Skehel *et al.*, 1977; Koff and Knight, 1979; Miller and Lenard., 1981).

The structure and function of the influenza virus genome has recently been extensively reviewed by

McCauley and Mahy (1983). Hence, only the salient points will be described.

Following uncoating the eight segments of vRNA are transcribed into two classes of RNA of positive polarity (Hay *et al.*, 1978; Hay and Skehel, 1979). One class serves as viral mRNA's and is composed of incomplete transcripts of vRNA which are polyadenylated at their 3'ends and possess a cap sequence at their 5'ends. The second class is composed of complete transcripts (cRNA), uncapped and non-polyadenylated which serve as templates for the replication of vRNA.

Transcription of RNA in infected cells is essentially divided into two distinct phases. The first phase, primary transcription (Flamand and Bishop, 1973), proceeds independently of protein synthesis and results in the production of mRNA complementary to each segment of RNA in close to equimolar quantities (Hay *et al.*, 1977[b]; Barrett *et al.*, 1979). Production of mRNA proceeds at a low level until approximately 90 minutes post infection after which it increases rapidly, probably as a result of the synthesis of new RNA polymerase molecules as this amplification does not occur in cells treated with cycloheximide (Barrett *et al.*, 1979; Hay *et al.*, 1981).

In contrast to the RNA polymerase complex associated with reovirions and other viruses that synthesise capped mRNA's, the RNA polymerase of purified influenza virus includes no capping enzymes. Instead, caps on influenza mRNA's are derived preformed from

heterologous capped cellular mRNA's (Bouloy *et al.*, 1979; Robertson *et al.*, 1980; Plotch *et al.*, 1981). This explains why transcription from cellular DNA is required for the synthesis of functional influenza mRNA (Barry *et al.*, 1962; Rott and Scholtissek, 1970; Mahy *et al.*, 1972).

A second phase of RNA synthesis (secondary transcription) is dependent upon protein synthesis (Barrett *et al.*, 1979; Hay *et al.*, 1980; Smith and Hay, 1982) and is necessary for the synthesis of template cRNA and for the subsequent synthesis of vRNA. The precise nature of the replicase responsible for this process is unknown. However, it seems likely that a modified form of the polymerase is involved (Barry and Mahy, 1979).

A scheme for influenza virus genome replication based upon models proposed by Hay *et al.*,(1980); Smith and Hay, (1982) and Ghendon *et al.*, (1982) is depicted in Fig. 1.2.2.ii. The relative rates of synthesis of each of the virus specified polypeptides upon cellular polysomes is controlled in such a way as to ensure that sufficient quantities of each protein are produced for the construction of progeny virus (Lamb and Choppin, 1976). Control is largely exerted at the level of replication although it can also occur at the level of cRNA and mRNA synthesis and also by splicing of mRNA's (Hay *et al.*, 1980; Smith and Hay, 1982; Ghendon *et al.*, 1982).

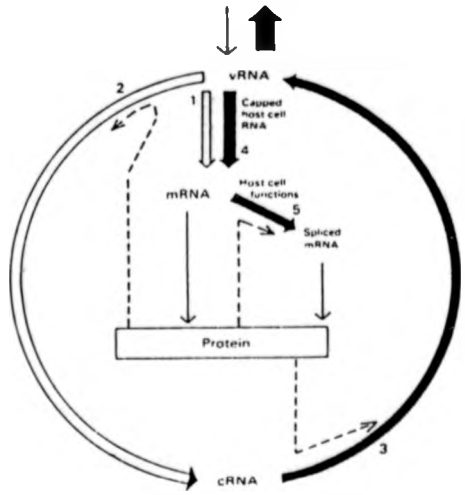


Fig. 1.2.2.ii Scheme for influenza genome replication.
⟹, transcription from input genome RNA;
➡, transcription from cRNA or newly synthesised vRNA;
----➤ necessary interactions.
Step 1: primary transcription, which occurs in the absence of protein synthesis but requires host cell transcription.
Step 2: cRNA synthesis from input vRNA.
Step 3: vRNA synthesis directed by cRNA.
Steps 2 and 3 both require ongoing protein synthesis. Step 4: transcription of newly synthesised vRNA to form mRNA requires host cell caps. Step 5: Modification of mRNA's 7 and 8 by splicing requires protein synthesis and undefined host cell functions.

(McCauley and Mahy, 1983)

Following synthesis, the virus polypeptides are transferred across the smooth and rough endoplasmic reticula where glycosylation of the haemagglutinin and neuraminidase takes place. The compounds D-glucosamine and 2-deoxy-D-glucose (Fig. 1.2.2.iii) have been shown to inhibit the glycosylation of virus proteins in

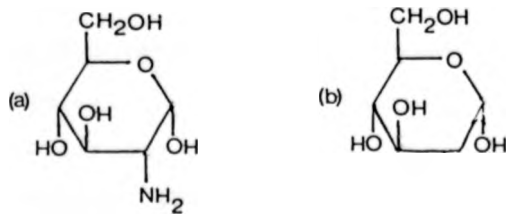


Fig. 1.2.2.iii D-Glucosamine (a) and 2-deoxy-D-glucose (b).

infected cells (Blough and Guitolini, 1979) which subsequently affects the functioning of these proteins (Chattergee *et al.*, 1979). The virus specified proteins then migrate to the cell periphery where the haemagglutinin and neuraminidase are inserted into the cell membrane. During the maturation process the haemagglutinin polypeptide is cleaved by a cellular protease into heavy HA_1 and light HA_2 (Waterfield *et al.*, 1979) which are linked together by a disulphide bond (Ward and Dophide, 1980; Waterfield *et al.*, 1981).

The other virus proteins accumulate beneath the membrane at the site of glycoprotein insertion and progeny virus particles containing a full complement of vRNA are produced by membrane budding (Landsberger *et al.*, 1971). The control mechanisms which ensure that each progeny virus particle contains a complete copy of the virus genome, probably arranged in a precise organised sequence, are unknown.

Neuraminidase appears to mediate the release of newly formed virus particles from the infected cell

(Bucher and Palese, 1975) and as such is a target for the design of inhibitors of influenza virus replication. The most potent inhibitor of neuraminidase discovered to date is 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA) (Fig. 1.2.2.iv) which has been shown to inhibit the replication of influenza virus in culture (Schulman and Palese, 1975). However, this compound has shown little activity in animal models.

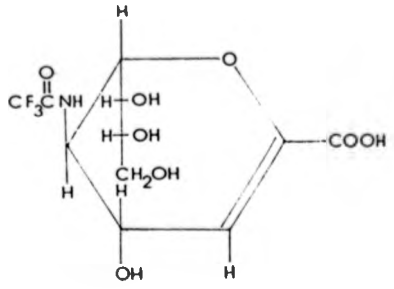


Fig. 1.2.2.iv FANA

1.2.3 The Herpesviruses

Herpesvirus is the generic name for a group of large DNA containing viruses (150-200 nm diameter) which have very similar structures but few other properties in common. Members of the group include herpes simplex virus (HSV) types 1 and 2, varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) all of which infect man, and numerous animal viruses which are found in a wide variety of species.

The herpesvirus genome is a linear double-stranded DNA molecule with a molecular weight of 100-150 x 10^6 (Roizman, 1979). This places the herpesviruses amongst the genetically most complex with sufficient nucleotides to code for some 70-80 different polypeptides. To date some 48 proteins have been identified in infected cells (Roizman *et al.*, 1974; Powell and Courtney, 1975), 24 of these being referred to as structural proteins. The genome is wound on a protein structure formed of basic histones (Furlong *et al.*, 1972), and is located in the centre of the nucleocapsid which measures 100 nm in diameter. The nucleocapsid exhibits icosahedral symmetry and is composed of 162 capsomeres (Wildy *et al.*, 1960) which take the form of elongated hollow prisms of dimensions 9.5 x 12.5 nm. Separating the nucleocapsid from the envelope is the tegument (Roizman and Furlong, 1974) which is composed of fibrous proteins. Its function is as yet unknown. The lipid skeleton of the envelope is derived from budding from the inner nuclear membrane of the infected cell (Roizman and Furlong, 1974) (Fig. 1.2.3). Seven virus-specified envelope glycoproteins (gA-gF and gY) have been found on the surface of the virion. At least one of which serves as a virus attachment protein (VAP) which recognises and binds to specific receptor sites on the surface of susceptible cells. Glycoprotein gB appears to be involved in the penetration of HSV and the oligosaccharide moiety of glycoprotein gC may play a role in modulating the hosts immune response to HSV.

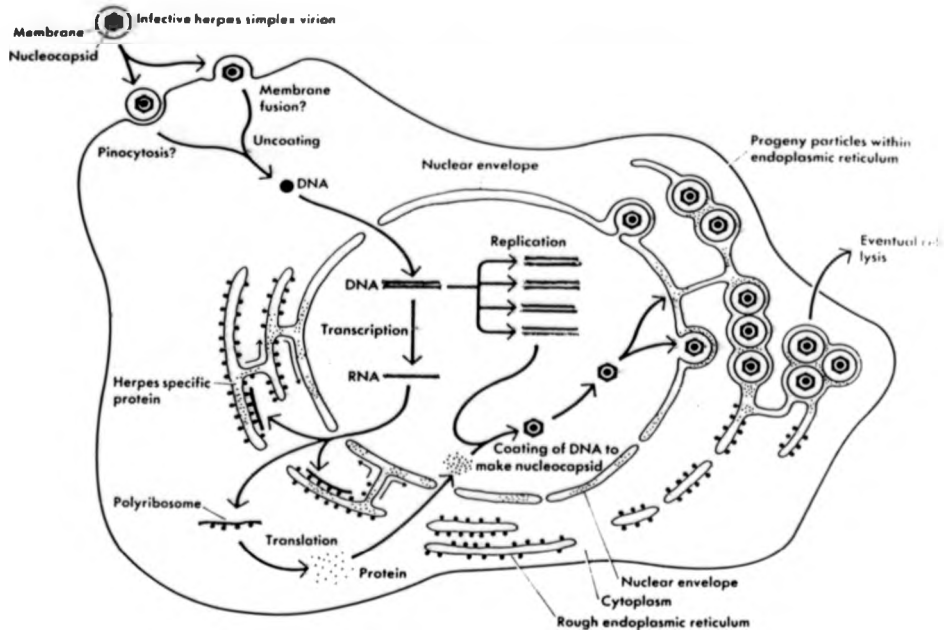


Fig. 1.2.3 Schematic representation of HSV replication in a permissive cell (from J. D. Watson, Molecular Biology of the Gene).

A highly schematic representation of HSV replication in a permissive cell is depicted in Fig. 1.2.3.

Infections by viruses of the herpes group differ from those caused by other viruses in that the infecting agent is not necessarily eliminated from the body on recovery. Instead, the virus may persist in a latent state in the dorsal root ganglia which innervate the initial site of infection. This persistent infection is asymptomatic but the virus may become reactivated and pass along the nerve trunks to the site of initial infection and cause a 'recurrent' infection,

which is often of a similar type to the initial infection.

1.2.4 Integration of Viral Nucleic Acid into the Host Genome

The replicative cycles outlined above are examples of acute viral infections in which the rapid replication of progeny virus is followed by its release from the infected cell in most cases accompanied by the death of the cell. However, some viral infections follow another replicative path which leads to the integration of viral genetic material into the host cells chromosomes. Each time the host cell divides the viral genes are duplicated along with the host's genes and as such the viral genes can be passed on to the host's offspring. Once integrated into the host's chromosomes, the activity of the viral genes is usually altered or totally suppressed. Such 'latent' infections can be reactivated in a number of diverse ways (e.g. u.v. radiation, carcinogens and other chemicals) to produce a sudden outbreak of the viral disease or transform the cell into a neoplastic state.

Little is known of the mechanisms which cause a particular virus to follow either the replicative or integrative paths. However, in the case of the retroviruses, integration into the host's chromosomes is an essential part of their life cycle, being necessary for the replication of the viral genome (Bishop, 1978) (Fig. 1.2.4).

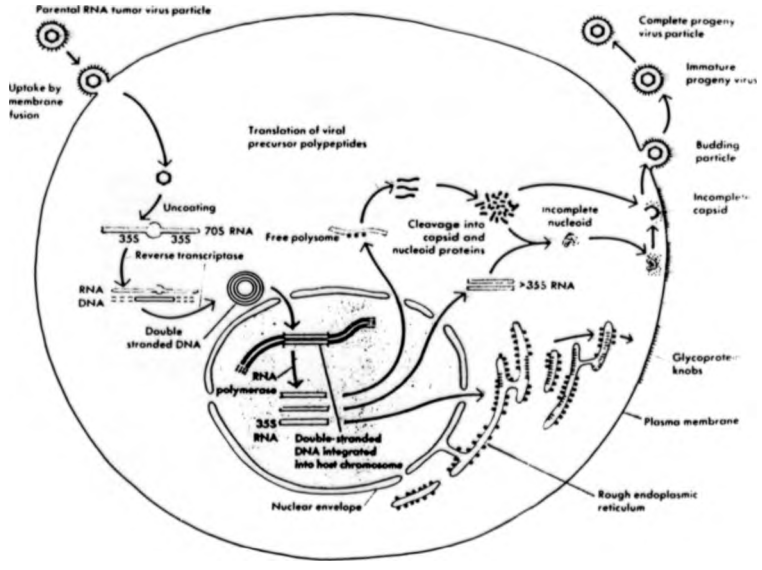


Fig. 1.2.4 Schematic diagram of the major steps in the replication of an RNA tumour virus. (Watson., 1976)

1.3 THE INTERFERON SYSTEM

In addition to a variety of host defences such as antibody production, cell mediated immunity and macrophage action, viruses also induce the production and release of interferons by certain cells (Isaacs and Lindenmann, 1957). These glycoproteins can in turn elicit the setting-up of an "antiviral state" in other host cells by means of the mechanism outlined in Fig. 1.3.i (Stewart, 1979). Two methods have been employed experimentally to increase the concentration of interferon in the body; the first and most obvious has been the administration of exogenous interferon.

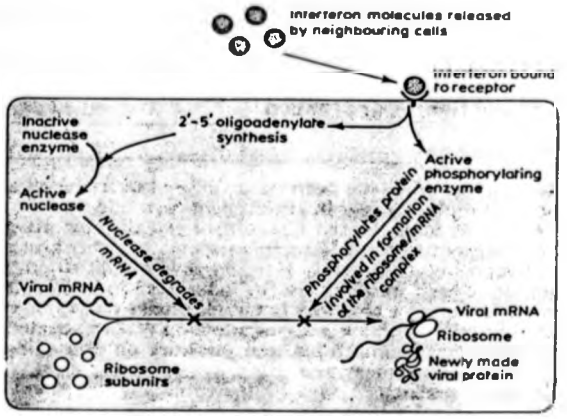


Fig. 1.3.i Two pathways by which interferon can inhibit viral protein synthesis. Viral double-stranded RNA activates both the phosphorylating enzyme and the enzyme responsible for 2',5'-oligoadenylate synthesis. Viral double-stranded RNA is also an initial inducer of interferon production. (Scott., 1983)

Research in this area has recently increased markedly by the possibility of producing large quantities of interferon using modern techniques, and initial clinical trials with interferon appear promising. The second mechanism to raise the bodily concentration of interferon has involved the administration of specific compounds such as double-stranded RNA with the aim of inducing the production of interferon (Kerr *et al.*, 1974). However, to date this approach has met with little success. Some of the antiviral activities of interferon are thought to be mediated by specific oligonucleotides which contain 2'-5' linkages rather than the normal

3'-5' linkages (Hovanessian *et al.*, 1977). These 2'-5' oligoadenylates (e.g. 5'-triphosphate of adenyl(2'-5')adenyl-(2'-5')adenosine (ppp(A^2p)$_2$A) (Fig. 1.3.ii) and higher oligomers)have a powerful effect on cellular protein synthesis at very low levels (Hovanessian and Wood, 1980) and therefore oligoadenylic acids related to this structure would appear to have potential as antiviral agents. However, problems associated with production, uptake and stability do not make them promising antiviral agents for clinical use at the present time (Hutchinson *et al.*, 1983).

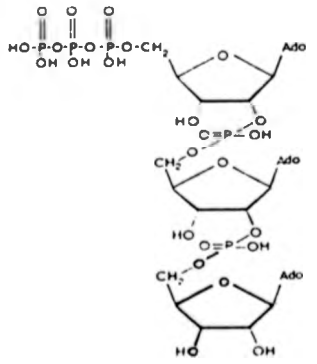


Fig. 1.3.ii ppp(A^2p)$_2$A

1.4 NEW ENZYMES PRESENT IN INFECTED CELLS

The main metabolic activity of a cell infected with an efficient virus is the synthesis of new viral proteins and nucleic acid. Some viruses, such as certain members of the parvovirus group are entirely reliant upon the enzymes of the host cell to perform both these tasks. However, it is now known that a large number of viruses contain enzymes which are distinct

from those of the host cell and/or carry the information in their genetic material for the synthesis in the infected cell of virus specific enzymes.

The basic premise concerning the intimate association between viral and cellular biosynthetic functions remains valid and even the most complex viruses depend at some stage on the proteins of the infected cell. The major reasons for the necessity of viruses to direct the synthesis of their own enzymes are outlined below.

(a) The synthesis of viral components may require a process which does not normally occur in the cell, e.g. all RNA viruses with a negative stranded genome contain an RNA dependent RNA polymerase since replication can occur only after transcription of the virus genome into mRNA (Baltimore *et al.*, 1970; Bishop *et al.*, 1971; Skehel, 1971) and there is no cellular enzyme which can carry out such a transcription.

(b) The virus replication cycle might involve a component which is not found in the cell, e.g. hydroxymethylcytosine in phage DNA (Mandel, 1968).

(c) A necessary cellular enzyme might be located at a site removed from that of the replicating virus, e.g. Pox viruses replicate in the cytoplasm and hence cannot utilise the cellular RNA polymerase which is located in the nucleus

(Katos and McAuslan, 1967; Schwartz and Dales, 1971).

(d) The cellular enzyme may be controlled so that it is specific for regions of macromolecules not present in the virus, e.g. σ mediated binding of *E. coli* RNA polymerase to regions of DNA.

(e) The cellular enzyme might have a very low activity, e.g. non-dividing animal cells have only very low levels of thymidine kinase activity and several DNA viruses code for the synthesis of this enzyme.

Enzyme activities detected in intact RNA virions or cells infected with RNA viruses are listed in Table 1.4.i and the DNA viruses and their associated enzyme activities in Table 1.4.ii. The lists contain enzymes for which the viral origin has not been proved conclusively (e.g. with the use of mutants) but is likely. Furthermore, the possibility must be borne in mind that some of the enzymes detected in enveloped virions may have a cellular origin.

1.4.1 Selective Inhibition of Viral Enzymes

One rational approach to drug design would be to select virus-specific processes and enzymes and then use whatever knowledge is available to design specific inhibitors (Mitchell, 1973; Becker, 1976). Assistance in this approach being provided by information

Table 1.4.i Enzymes Induced by RNA Viruses

Virus	Enzyme activity
Picornaviruses	
Poliovirus, EMC, FMDV, rhinovirus	RNA-dependent RNA polymerase, EC 2.7.7
Poliovirus, FMDV	endonuclease, EC 3.1.4
Poliovirus, EMC, FMDV	peptide hydrolase (protease), EC 3.4
Reoviruses	
Reovirus	(guanine-7)-methyltransferase, EC 2.1.1
Reovirus, rotavirus	RNA-dependent RNA polymerase, EC 2.7.7
Reovirus	poly G polymerase, EC 2.7.7
Reovirus	poly A polymerase, EC 2.7.7
Reovirus	mRNA guanylyltransferase, EC 2.7.7
Reovirus	nucleoside triphosphatase, EC 3.6.1
Reovirus	polynucleotide 5'-triphosphatase, EC 3.6.1
Reovirus	triphosphate-pyrophosphate exchange
Togaviruses	
Sindbis virus, Semliki Forest virus	protein kinase, EC 2.7.1
Sindbis virus, Semliki Forest virus	RNA-dependent RNA polymerase, EC 2.7.7
Sindbis virus	endonuclease, EC 3.1.4
Orthomyxoviruses	
Influenza A virus	protein kinase, EC 2.7.1
Influenza A virus	nucleoside diphosphate kinase, EC 2.7.4
Influenza A, B, C virus	RNA-dependent RNA polymerase, EC 2.7.7
Influenza A, B, virus	neuraminidase, EC 3.2.1
Influenza A virus	nucleoside triphosphatase, EC 3.6.1
Paramyxoviruses	
NDV	(guanine-7)-methyltransferase, EC 2.1.1
Sendai virus	protein kinase, EC 2.7.1
NDV, mumps virus, measles virus, Sendai virus, SV5	RNA-dependent RNA polymerase, EC 2.7.7
NDV, Sendai virus, SV5	poly A polymerase, EC 2.7.7
NDV	mRNA guanylyltransferase, EC 2.7.7
Sendai virus	endonuclease, EC 3.1.4
NDV, mumps virus, Sendai virus	neuraminidase, EC 3.2.1
NDV	adenosine triphosphatase (ATPase), EC 3.6.1
Rhabdoviruses	
VSV	(guanine-7)-methyltransferase, EC 2.1.1
VSV	(adenine-2)-methyltransferase, EC 2.1.1
VSV, rabies virus	protein kinase, EC 2.7.1
VSV	nucleoside diphosphate kinase, EC 2.7.1
VSV, rabies virus, salmonid viruses	RNA-dependent RNA polymerase, EC 2.7.7
VSV	mRNA guanylyltransferase, EC 2.7.7
VSV	poly A polymerase, EC 2.7.7
VSV	endonuclease, EC 3.1.4
VSV	nucleoside triphosphatase, EC 3.6.1
Retroviruses	
ASV, MuLV, MTV, RSV, FeLV, AMV	protein kinase, EC 2.7.1
RSV, AMV, etc.	RNA-dependent DNA polymerase, (reverse transcriptase), EC 2.7.7
RSV, AMV, etc.	DNA-dependent DNA polymerase, (reverse transcriptase), EC 2.7.7
AMV, RSV	endonuclease, EC 3.1.4
RLV	RNase H, EC 3.1.4
RLV	protease, EC 3.4
AMV	nucleoside triphosphatase, EC 3.6.1
Arenaviruses	
Pichinde virus	RNA-dependent RNA polymerase, EC 2.7.7
Bunyaviruses	
Uukuniemi virus	RNA-dependent RNA polymerase, EC 2.7.7

Avian myeloblastosis virus, AMV; avian sarcoma virus, ASV; encephalomyocarditis virus, EMC; feline leukemia virus, FeLV; foot and mouth disease virus, FMDV; mammary tumor virus, MTV; murine leukemia virus, MuLV; Newcastle disease virus, NDV; Raucher leukemia virus, RLV; Rous sarcoma virus, RSV; simian virus 5, SV5; vesicular stomatitis virus, VSV.

(Helgstrand and Oberg., 1980)

Table 1.4.ii Enzymes Induced by DNA Viruses

Virus	Enzyme activity
Parvoviruses	
Kilham rat virus	DNA-dependent DNA polymerase, EC 2.7.7
Papovaviruses	
Polyoma virus	protease, EC 3.4
Adenoviruses	
Adenovirus type 2	protein kinase, EC 2.7.1
Adenovirus type 2	protease, EC 3.4
Herpesviruses	
HSV-1, HSV-2	ribonucleotide reductase, EC 1.17.4
HSV-1, pseudorabies virus	protein kinase, EC 2.7.1
HSV-1, HSV-2, VZV, EBV, pseudorabies virus, HVT, EHV-1	nucleoside phosphotransferase (thymidine kinase), EC 2.7.1
HSV-1	thymidylate kinase, EC 2.7.4
HSV-1, HSV-2, VZV, HVT, MDV, CMV, EBV, pseudorabies virus, EHV-1	DNA-dependent DNA polymerase, EC 2.7.7
HSV-1, HSV-2, VZV, CMV, EBV	DNase, EC 3.1.4
HSV-1	Deoxycytidine deaminase, EC 3.5.4
Iridoviruses	
Frog virus 3	protein kinase, EC 2.7.1
ASFV	DNA-dependent DNA polymerase, EC 2.7.7
Frog virus 3	DNase, EC 3.1.4
Frog virus 3	RNase, EC 3.1.4
Frog virus 3	adenosine triphosphatase (ATPase), EC 3.6.1
Poxviruses	
Vaccinia virus	(guanin-7)-methyltransferase, EC 2.1.1
Vaccinia virus	mRNA ribose-2′-*O*-methyltransferase, EC 2.1.1
Vaccinia virus	protein kinase, EC 2.7.1
Vaccinia virus	nucleoside phosphotransferase (thymidine kinase), EC 2.7.1
Vaccinia virus, rabbit pox virus	DNA-dependent RNA polymerase, EC 2.7.7
Vaccinia virus	DNA-dependent DNA polymerase, EC 2.7.7
Vaccinia virus	poly A polymerase, EC 2.7.7
Vaccinia virus	mRNA guanylyltransferase, EC 2.7.7
Vaccinia virus	DNase, EC 3.1.4
Vaccinia virus	nucleoside triphosphatase, EC 3.6.1
Vaccinia virus	polynucleotide 5′-triphosphatase, EC 3.6.1
Vaccinia virus	polynucleotide ligase, EC 6.5.1
Hepatitis B viruses	
Human hepatitis B virus, woodchuck hepatitis B virus	DNA-dependent DNA polymerase, EC 2.7.7

African swine fever virus, ASFV; cytomegalo virus, CMV; Epstein-Barr virus, EBV; equine herpesvirus type 1, EHV-1; herpes simplex virus type 1, HSV-1; herpes simplex virus type 2, HSV-2; herpes-virus of turkeys, HVT; Marek's disease virus, MDV; varicella-zoster virus, VZV.

(Helgstrand and Oberg., 1980)

acquired on the general structures and in some cases modes of action, of compounds possessing antiviral activity. Another rational approach, the selective activation approach, involves searching for a compound which is a good substrate for the viral enzyme alone. This compound is then activated by that enzyme to produce an inhibitor of viral multiplication but only in infected cells (Cheng *et al.*, 1979).

Viral enzymes can in general be regarded as isoenzymes of cellular enzymes. Although isoenzymes utilise identical substrates they can show considerable differences in their sensitivities to inhibitors. The classical example being the highly sensitive nature of bacterial and plasmodial dihydrofolate reductases to trimethoprim which shows virtually no activity against the human isoenzyme. A number of reviews concerning viral enzymes and inhibitors thereof have been published (Helgstrand and Öberg, 1980; Smith *et al.*, 1980) and details on similar cellular enzymes can be found in standard works on enzymology.

1.4.2 Nucleoside Analogues as Antiviral Agents

A large number of the antiviral compounds developed to date are effective against herpesviruses and the antiviral specificity of these compounds largely relies on two virus induced enzymes, thymidine-(deoxycytidine) kinase(TK) and DNA polymerase.

Herpesvirus TK will accept a much broader

range of substrates than will the cytoplasmic and mitochondrial TK's (De Clerq *et al.*, 1977; Dobersen and Greer, 1978; Cheng *et al.*, 1979) and phosphorylate them to the corresponding 5'-phosphates. The phosphorylated nucleoside analogues subsequently effect virus replication by inhibiting the viral or cellular processes which support it (Fig. 1.4.2).

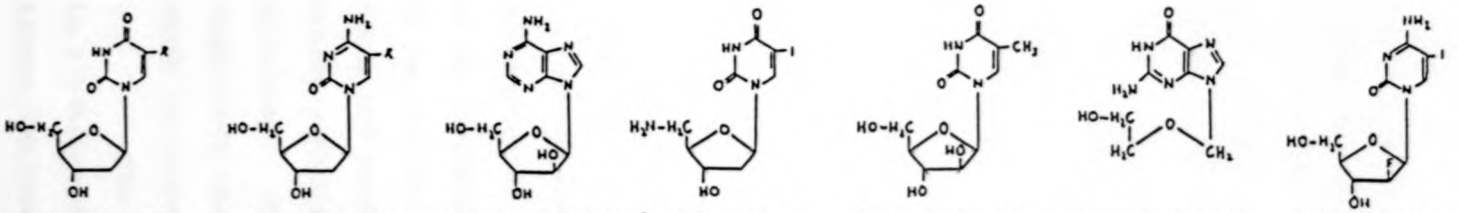
1.4.2.1 5-Iodo-2'-deoxyuridine (Idoxuridine, 5-iodo-dUrd) (Fig. 1.4.2) is phosphorylated in virus-infected and non-infected cells and is subsequently incorporated into both viral and cellular DNA (Prusoff and Goz, 1973) where it interferes with the process of replication and transcription. A number of analogues of 5-iodo-dUrd (e.g. 5-ethyl-, 5-propyl-dUrd) do show selectivity against herpesviruses (De Clerq *et al.*, 1978) probably due to a selective phosphorylation by the viral TK and eventual incorporation into the DNA of virus-infected cells.

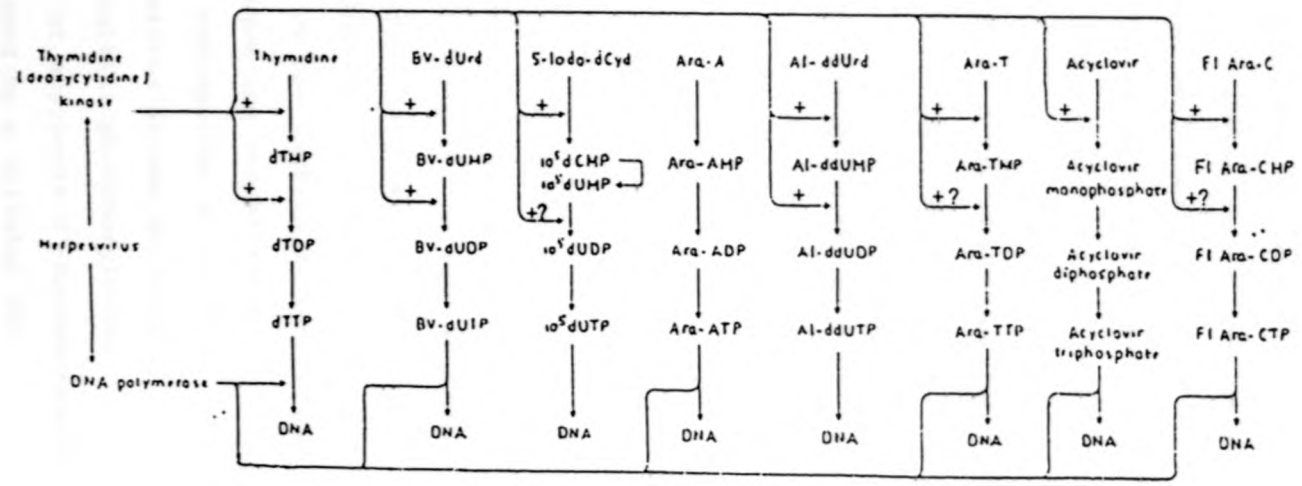
1.4.2.2 E-5-(2-Bromovinyl)-2'-deoxyuridine (BV-Urd) (Fig. 1.4.2) and other E-5-(2-halogenovinyl)-dUrd derivatives (De Clerq *et al.*, 1979; 1980a) are specifically phosphorylated by the herpesvirus TK (Cheng *et al.*, 1981), which restricts their action to the virus infected cell. BV-dUrd is a very potent inhibitor of HSV-1 but is less active against HSV-2 possibly because HSV-1 TK phosphorylates BV-dUrd to the 5'-mono- and diphosphates whereas HSV-2 TK does not phosphorylate beyond the monophosphate stage (Descamps

Fig. 1.4.2 Role of virus-induced thymidine(deoxycytidine) kinase and DNA polymerase in the selective anti-herpes activity of BV-dUrd, 5-iodo-dCyd, AraA, AI-ddUrd, Ara-T, Acyclovir and FIAra-C.

For BV-dUrd, Ara-T, acyclovir and FIAra-C the selective antiherpes activity depends on both thymidine(deoxycytidine) kinase and DNA polymerase for AI-ddUrd and 5-iodo-dCyd it depends on virus-induced thymidine-(deoxycytidine) kinase only, and for Ara-A it depends on viral DNA polymerase only. In addition to the indicated pathway, FIAra-C may also be metabolised to the 5'-mono-, di-, and triphosphates of FIAra-U, FAra-U, FAra-C and FAra-T and these 5'-triphosphates may interact with DNA polymerase and eventually be incorporated into DNA.

(Adapted from De Clerq, 1982)


R = I, 5-Iodo-2'-deoxyuridine (5-Iodo-dUrd)
R = CF3, 5-Trifluoromethyl-2'-deoxyuridine (5-CF3-dUrd)
R = (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BV-dUrd)
R = I, 5-Iodo-2'-deoxycytidine (5-Iodo-dCyd)
9-β-D-Arabinofuranosyladenine (Ara-A)
5'-Amino 5 Iodo-2' 5'-Dideoxy uridine (AI-ddUrd)
1-β-D-Arabinofuranosylthymine (Ara-T)
9 (2-Hydroxyethoxymethyl) guanine acyclovir
2' Fluoro-5-iodo 1-β-D arabinofuranosylcytosine (FIAra-C)


Thymidine (deoxycytidine) kinase
Herpesvirus
DNA polymerase
Thymidine
dTMP
dTDP
dTTP
BV-dUrd
BV-dUMP
BV-dUDP
BV-dUTP
5-Iodo-dCyd
10^5dCMP
10^5dUMP
10^5dUDP
10^5dUTP
Ara-A
Ara-AMP
Ara-ADP
Ara-ATP
AI-ddUrd
AI-ddUMP
AI-ddUDP
AI-ddUTP
Ara-T
Ara-TMP
Ara-TDP
Ara-TTP
Acyclovir
Acyclovir monophosphate
Acyclovir diphosphate
Acyclovir triphosphate
FI Ara-C
FI Ara-CMP
FI Ara-CDP
FI Ara-CTP
DNA

and De Clerq, 1981) further phosphorylation by cellular enzymes yields the 5'-triphosphate which interacts with the viral DNA polymerase to a far greater extent than with the cellualr enzyme (Allaudeen *et al.*, 1981). BV-dUrd can also be incorporated into the DNA of virus-infected cells (Allaudeen *et al.*, 1981) but the contribution which this makes to antiviral activity has not been determined. Initial clinical results with BV-dUrd are very promising (Maugdal *et al.*, 1981; De Clerq *et al.*, 1980b).

1.4.2.3 9-(2-Hydroxyethoxymethyl)guanine (Acyclovir) (Fig. 1.4.2) As with other selective antiherpes drugs acyclovir does not become active until it has been specifically phosphorylated by a viral TK (Elion *et al.*, 1977; Fyfe *et al.*, 1978). Analysis of herpesviruses which are susceptible to acyclovir suggests that it is a deoxycytidine kinase activity which is responsible for its initial phosphorylation. For HSV-1, HSV-2 and VZV the deoxycytidine kinase activity resides on the same molecule as the thymidine kinase activity. However, the TK's of pseudorabies virus, infectious bovine rhinotracheitis virus and herpesvirus saimiri do not possess deoxycytidine kinase activity and these viruses are resistant to acyclovir. These viruses are susceptible to BV-dUrd suggesting that it is the thymidine kinase activity which is responsible for its initial phosphorylation.

The phosphorylation of acyclovir 5'-monophosphate to 5'-diphosphate is accomplished by a cellular GMP kinase (Miller and Miller, 1980) and as its 5'-triphosphate

it reacts specifically with the viral DNA polymerase (Furman *et al.*, 1979). Acyclovir is also incorporated into DNA where it acts as a chain terminator as it does not have the 3'-OH necessary for chain elongation (Furman *et al.*, 1980). Acyclovir has been shown to be clinically effective against a number of herpesvirus infections (Peterslund *et al.*, 1981; Saral *et al.*, 1981; Selby *et al.*, 1981).

1.4.2.4 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine (Ribavirin) (Fig. 1.4.2.4) has been shown to be effective against a broad range of both RNA and DNA viruses (Smith and Kirkpatrick, 1980). It can be

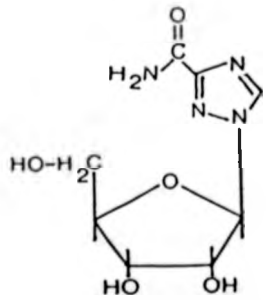


Fig. 1.4.2.4 Ribavirin

converted to the mono-, di- and triphosphate forms by cellular kinases (Miller *et al.*, 1977). As the 5'-monophosphate form it inhibits inosine 5'-monophosphate (IMP) dehydrogenase which is an essential enzyme in the biosynthesis of GTP (Streeter *et al.*, 1973). The biosynthesis of dTTP is also inhibited although the exact mechanism by which this is achieved remains

unknown (Drach *et al.*, 1981). It is this inhibition of nucleotide biosynthesis which has been assumed to be largely responsible for the antiviral activity of ribavirin.

Ribavirin 5'-triphosphate has been shown to inhibit mRNA guanyltransferase which transfers GMP from GTP to the 5'-terminus of acceptor mRNA (Goswami *et al.*, 1979). However, since both cellular and viral mRNA's (except picornaviral mRNA's) require capping it is difficult to envisage how this could result in antiviral specificity. Ribavirin 5'-triphosphate is a selective inhibitor of influenza RNA polymerase (Eriksson *et al.*, 1977). During the transcription of influenza RNA 5'-cap sequences are cleaved from cellular mRNA's to provide primers for viral RNA (Bouloy *et al.*, 1978; Plotch *et al.*, 1979). The selective inhibition of influenza RNA polymerase by ribavirin 5'-triphosphate could well be due to an inhibition of the 5'-cap transfer reaction (Eriksson *et al.*, 1977), and could explain the relatively specific activity of ribavirin against influenza virus although concomitant effects by inhibition of nucleoside biosynthesis should also be considered.

Ribavirin has been shown to be effective in experimental influenza A infection of mice but the compound produced no beneficial effects when administered by the oral route to humans during an outbreak of influenza A (Smith *et al.*, 1980).

1.5 INHIBITORS OF VIRAL NUCLEIC ACID POLYMERASES

Several nucleoside analogues have antiherpes activity based upon their selective activation by a herpesvirus thymidine (deoxycytidine) kinase activity. However, one clinically important herpesvirus, CMV, does not induce such an activity in infected cells (Zavada *et al.*, 1976) and furthermore, outside of the herpesvirus group only vaccinia has been shown to code for such an activity (Table 1.4.ii). Consequently, the antiviral spectrum of these nucleoside analogues is restricted and as such they do not provide the broad-spectrum antiviral 'penicillin'.

The virus-specific nucleic acid polymerases are accessible for nearly all viruses (Table 1.4.i and 1.4.ii), are easily assayed in cell-free systems and afford good primary targets for antiviral agents.

The process of nucleic acid biosynthesis can be broken down into four distinct steps (Fig. 1.5).

(a) Template binding The polymerase recognises and binds to a specific nucleotide sequence on the template nucleic acid.

(b) Initiation A nucleoside 5'-triphosphate or primer oligonucleotide binds to the enzyme template complex at a site corresponding to the 5'-terminus of the nucleic acid to be synthesised. (The order of (a) and (b) has not been established.)

(c) Elongation Nucleoside 5'-triphosphates

are added sequentially, in an order determined by the template, to the 3' end of the nascent nucleic acid with elimination of pyrophosphate.

(d) Termination On reaching a specific oligonucleotide sequence on the template, synthesis of nucleic acid ceases and the complex of template, polymerase and nascent nucleic acid dissociates.

Interference with any one of these processes would result in an inhibition of nucleic acid polymerisation. However, if it is to be selectively inhibited, the viral polymerase must contain a different amino acid sequence from that of the host polymerase at an essential site on the enzyme. Suitable starting points for the design of

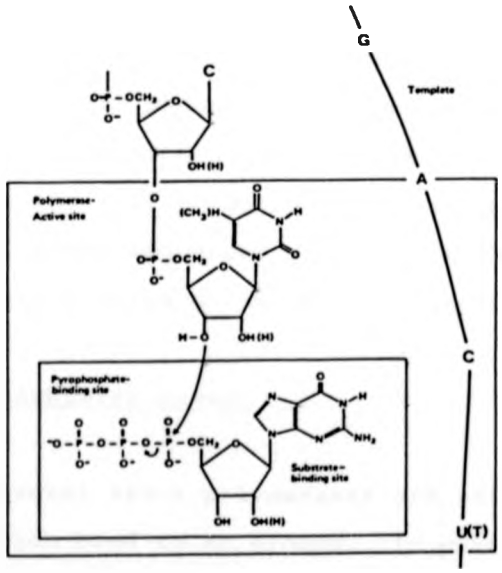


Fig. 1.5 Elements of nucleic acid synthesis (from Helgstrand and Öberg, 1980).

inhibition would be the substrates (discussed in Section 1.4.2) and the by-products of the reaction. Viral templates or primers are also useful targets as are other essential elements of nucleic acid synthesis such as sulphydryl groups, other amino acids and metal ions.

1.5.1 Template and Primer Binding

Oligo(deoxy)ribonucleotides which have a structure complementary to a nucleotide sequence on the viral nucleic acid would be expected to hybridise with the viral nucleic acid and, depending upon its nature, inhibit replication, transcription or translation. The tridecanucleotide d(A-A-T-G-G-T-A-A-A-A-T-G-G) has a sequence complementary to the 3'-5'-terminal sequence of the Rous sarcoma virus genome and has been shown to inhibit both virus production and cell transformation (Zamecnik and Stephenson, 1978). To determine the full potential of this approach information on the sequences of viral DNA and RNA primer and polymerase binding sites will be required.

1.5.2 Sulphydryl Groups

Several viral polymerases are affected by compounds which bind to SH groups. In particular, Billard and Peets (1974) reported the inhibition of influenza virus RNA polymerase by selenocystine, 4-(2-propinyloxy)-β-nitrostyrene and acetylaranotin,

and reverse transcriptase is inhibited by gliotoxin and β-lapachone (De Clerq *et al.*, 1978; Schuerch and Wehrli, 1978).

1.5.3 Other Amino Acid Groups

Reverse transcriptases contain an argininyl residue which is responsible for binding of the template primer to the active site (Borders *et al.*, 1975). Pyridoxal phosphate forms a Schiff's base with this essential amino acid and is a strong competitive inhibitor of this class of viral enzymes (Modak, 1976).

1.5.4 Metal Ions

Oxford and Perrin (1974) detected the presence of zinc in highly purified preparations of influenza virus and have shown that many compounds which form very stable complexes with 'soft' heavy metal ions such as zinc (Pearson, 1966) are effective inhibitors of influenza RNA polymerase. The hypothesis has been postulated that these compounds inhibit the enzyme by forming an enzyme-zinc-ligand complex (Oxford and Perrin, 1974). Complexes formed from chelating agents and free metal ions in solution have also been shown to affect polymerases (Levinson *et al.*, 1973). In general, however, simple chelating agents are not expected to exhibit any specificity.

1.5.5 Pyrophosphate Analogues

Pyrophosphate (17) is a by-product of the polymerisation reaction (Fig. 1.5). Consequently, analogues of pyrophosphate might be expected to bind to a pyrophosphate binding site of the polymerase along the forward reaction coordinate or alternatively such compounds might reverse the polymerisation reaction resulting in a breakdown of nucleic acid. In either case the net result would be an inhibition of normal polymerase activity.

Such reasoning could have led to the discovery of phosphonoacetic acid (PAA (1) Fig. 1.5.5). However, the antiviral activity of PAA was discovered by random

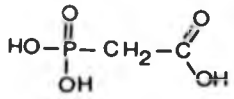


Fig. 1.5.5 Phosphonoacetic acid (PAA) (1)

screening of herpesvirus-infected WI-38 tissue culture cells (Shipkowitz *et al.*, 1973). Consistent antiviral activity required a high concentration of drug (100 µg/ml) but the drug was tolerated well showing little or no effect on cell growth or division at levels several times that required to inhibit herpesvirus replication. The drug also appeared to have some potential as a clinical agent being effective against experimental herpesvirus

infections of mice and rabbits.

These initial results prompted studies on the effect of PAA on molecular events in normal and HSV-infected human cells. Overby *et al.* (1974) showed that PAA did not produce any signi ficant changes in the rate of DNA, RNA or protein synthesis in normal cells but in HSV-infected cells PAA completely inhibited the production of virus-specific DNA. Mao and co-workers (1975) extended this work by isolating the HSV-induced DNA polymerase from infected cells and demonstrated that PAA affects the synthesis of virus-specific DNA by a direct inhibition of this enzyme.

In an attempt to understand the antiviral activity of PAA it is helpful to compare the properties and functions of the normal cellular DNA polymerases to those of the herpesvirus-induced DNA polymerase.

1.6 CELLULAR AND HERPESVIRUS - INDUCED DNA POLYMERASES

In many ways the DNA polymerase activities induced by herpesviruses are similar to the mammalian DNA polymerase α of uninfected cells (Table 1.6.i). The synthetic primer-templates $(dG)\approx_{15}\text{-}(dC)_n$ and $(dT)\approx_{15}\text{-}(dA)_n$ as well as double stranded DNA "activated" by digestion with pancreatic DNase are all copied efficiently by both enzymes (Wagner *et al.*, 1974; Weissbach *et al.*, 1973). The enzymes also have similar molecular weights and cannot be resolved on sucrose gradients (Hirai and Watanabe, 1976) and furthermore both enzymes are inhibited by N-ethyl

Table 1.6.i Properties and Functions of Cellular and Herpesvirus - Induced DNA Polymerases

DNA Polymerase	M. Wt.	Function	pH Optimum	Salt Optimum	MalNet Inhibition	Preferred Primer/Template	[PAA] (μM) Producing 50% inhibition	Reference
Cellular α	1.1 to 1.8 x 10^4	Cellular DNA replication	7.3	20 mM KCl	Very sensitive	'Activated' DNA	25-30	Weissbach *et al.*, 1971 Sabourin *et al.*, 1978 Eriksson *et al.*, 1982
Cellular β	3.5 x 10^4	Cellular DNA repair	7.9	0	Resistant	$(dT)_{\approx 15} \cdot (dA)_n$	> 100	Wang *et al.*, 1975 Sabourin *et al.*, 1978
Cellular γ	1.1 to 3.3 x 10^4	Mitochondrial DNA Synthesis	8.5	100 mM KCl	Mod. sensitive	$(dT)_{\approx 15} \cdot (dA)_n$	> 100	Knopf *et al.*, 1976
HSV-type 1	1.5 x 10^4 1.8 to 2.0 x 10^4	Viral DNA replication	8.0	150 mM K_2SO_4	Sensitive	$(dG)_{12} \cdot (dC)_n$	0.4 to 7	Weissbach *et al.*, 1973 Powell and Purifoy, 1977 Eriksson *et al.*, 1980
HSV-type 2	1.5 x 10^4	Viral DNA replication	8.0	100 mM $(NH_4)_2SO_4$	ND	'Activated' DNA	1 to 2	Powell and Purifoy, 1977
Human CMV	ND	Viral DNA replication	8.0	120 mM KCl	Sensitive	$(dT)_{\approx 15} \cdot (A)_n$	0.4	Huang, 1975 Ooka *et al.*, 1979 Eriksson *et al.*, 1982
Epstein-Barr Virus	ND	Viral DNA replication	7.5	50 mM $(NH_4)_2SO_4$	ND	$(dG)_{\approx 15} \cdot (dC)_n$	~ 10 μg/ml	Thorley-Lawson and Strominger, 1976 Hirai and Watanabe, 1976 Grossberger and Clough, 1982

*DNA polymerase β from Novikoff hepatoma has been described sensitive to MalNet (Stalker *et al.*, 1976)

ND Not determined

maleimide (MalNet) (Wagner *et al.*, 1974; Weissbach *et al.*, 1973). However, the virus-induced enzymes do differ from the cellular enzyme in some important respects (Mar and Huang, 1974).

The herpesvirus-induced enzymes can be separated from cellular DNA polymerase α by phosphocellulose chromatography (Weissbach *et al.*, 1973). With the exception of the DNA polymerase isolated from cells infected with Marek's disease virus (Boezi *et al.*, 1974) all the virus-induced enzymes studied to date are most active at high ionic strength. At such salt concentrations the host cell polymerases are almost totally inactive (Weissbach *et al.*, 1973). However, the most specific and useful method of classification is the reactivity of virus-induced and cellular polymerases to specific anti-sera (Table 1.6.ii). Sensitivity of herpesvirus-induced but not cellular DNA polymerases to PAA has been used to distinguish between these enzymes but it is now clear that this specificiity is not absolute. DNA Polymerase α but not β or γ is inhibited by PAA, however, it is 15 to 30 times less sensitive than the herpesvirus-induced enzymes (Bolden *et al.*, 1975; Miller and Rapp, 1976; Sabourin *et al.*, 1978). The isolation and characterisation of PAA resistant herpesvirus strains, (P^r), by Honess and Watson (1977) and Powell and Purifcy (1977) have not only provided conclusive evidence that PAA inhibits herpesvirus replication by a direct interaction with the virus-induced DNA polymerase but have also cast further doubts on the validity of classifying a polymerase solely

Antibody against	Assay system	Specificity		Reference
		Cross with	No Cross with	
DNA polymerase α	Enzyme neutralisation	DNA polymerase α	DNA polymerase β or γ	Spandari *et al.*, 1974; Smith *et al.*, 1975
DNA polymerase γ	Enzyme binding assay	DNA polymerase γ	DNA polymerase α or β	Robert-Guroff and Galb, 1977
Herpesvirus-infected cells or purified polymerase	Enzyme neutralisation	Strongly with herpesvirus-induced DNA polymerase. Shows specificity among different herpesviruses	DNA polymerase α or β	Weissbach *et al.*, 1973; Allen *et al.* 1977

Table 1.6.ii Specificities of antibodies against different DNA polymerases

on its sensitivity to PAA. However, a combination of a number of the above criteria should lead to an unequivocal identification of polymerase type.

1.6.1 Structure-Activity Relationships

The simple chemical structure of PAA provides little opportunity for optimisation of antiviral activity by chemical manipulation. However, a number of analogues have been synthesised and tested as inhibitors of isolated herpesvirus-induced DNA polymerase and herpesvirus replication in cell culture and animal models (Table 1.6.1).

Herrin *et al.* (1977) reported the inhibition of HSV-1-induced DNA polymerase by low molecular weight carboxyl esters of PAA but these results have been questioned by Boezi (1979) who found such compounds to be inactive. This discrepancy has yet to be clarified but in general, esters of PAA are inactive.

Substitution of the phosphono group by carboxyl and sulphono groups results in an abolition of activity against the herpesvirus of turkeys induced DNA polymerase (Leinbach *et al.*, 1976). Replacement of the phosphono and/or carboxyl groups by arseno groups yields the compounds methylenediarsonate and arsenomethyl phosphonate, which Newton (1979) reports as being equally effective as PAA in plaque reduction assays involving HSV-1. She further states that the antiviral activity of these compounds was short-lived possibly due to their instability. However, Overby (1982) points out that organic arsenicals are normally very stable and postulates that the observed

Analog	Reference
I. Carboxyl esters	
Ethyl; propyl; *t*-butyl	Herrin et al. (1977)
Cyclohexyl; octyl; benzyl	Herrin et al. (1977)
II. Monophosphate esters	
Methyl; propyl; hexyl	Herrin et al. (1977)
III. Triesters	
Trimethyl	Lee et al. (1976)
Triethyl	Shipkowitz et al. (1973)
IV. Monophosphinic acids	
Phenyl; 4-methoxyphenyl; methyl	Herrin et al. (1977)
V. Phosphono analogs	
Sulfoacetate	Leinbach et al. (1976)
Malonate	Leinbach et al. (1976)
Arsenoacetate	Newton (1979)
VI. Carboxyl analogs	
Phosphonoacetaldehyde	Boezi (1979)
Phosphonoacetamide	Boezi (1979)
N-Methylphosphonoacetamide	Boezi (1979)
N-Propyl-; *N*-butyl-; *N*-cyclohexyl-; *N*-amantylphosphonoacetamide	von Esch (1978)
Acetonylphosphate	Boezi (1979)
Aminomethylphosphonate	Lee et al. (1976)
α-Amino ethyl phosphonate	Lee et al. (1976)
N-(phosphonoacetyl)-L-aspartate	Boezi (1979)
Methylene diphosphonate	Lee et al. (1976)
Methylene diarsonate	Newton (1979)
Arsonomethylphosphonate	Newton (1979)
VII. Methylene analogs	
Phosphonoformate	Reno et al. (1978)
Phosphonopropionate	Shipkowitz et al. (1973)
Phosphonobutyrate	Shipkowitz et al. (1973)
α-Phosphonopropionate	Leinbach et al. (1976)
α-Methyl-2-phosphonopropionate	Leinbach et al. (1976)
α-Phenylphosphonoacetate	Leinbach et al. (1976)
α-Aminophosphonoacetate	Leinbach et al. (1976)
VIII. Other related compounds	
Phosphoglycolate	Leinbach et al. (1976)
Imidodiphosphonate	Boezi (1979)
Carbamyl phosphate	Boezi (1979)
2'-Deoxyribothymidine-5'-phosphorophosphonoacetate	Boezi (1979)
Purine-5'-mono-carboxymethyl-phosphonate	Heimer and Nussbaum (1977)
Pyrimidine-5'-mono-carboxymethyl-phosphonate	Heimer and Nussbaum (1977)

Table 1.6.1 Analogues of phosphonoacetate tested for antiherpesvirus activity (taken from L. R. Overby, 1982).

activity of these aresno-analogues may be a manifestation of their cytotoxicity. Inhibition studies involving isolated virus-induced DNA polymerase are obviously needed to help delineate the true antiviral nature of these compounds. Substitution of the carboxyl group by moieties other than arseno generally result in an abolition of activity. The only exceptions to this rule are certain amide derivatives described by Von Esch (1978), but it would appear that a free carboxyl group is necessary for optimal activity.

Pyrimidine and purine nucleoside analogues of PAA have been shown to be effective against HSV infections in mice and cell culture but the mechanism of action has not been shown to involve a direct interaction with the virus-induced DNA polymerase (Heimer and Nussbaum, 1977).

Several studies have revealed that separation of phosphono and carboxyl groups is not necessary for antiherpesvirus activity as phosphonoformate (PFA(2) Fig. 1.6.1) is as equally effective as PAA (Reno *et al.*, 1978). However, increasing the carbon chain length to produce the propionate and butyrate analogues or substitution on the methylene carbon of PAA by methyl,

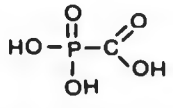


Fig. 1.6.1 Phosphonoformic acid (PFA)(2).

amino or phenyl groups results in an abolition of anti-herpesvirus activity in cell culture (Shipkowitz *et al.*, 1973; Leinbach *et al.*, 1976).

The study of analogues of PAA has revealed that the structural requirements for antiherpesvirus activity are rigorous and that an unsubstituted phosphono group and an unsubstituted carboxyl group either linked to one another directly or by an unsubstituted methylene group are required for optimal activity.

1.6.2 Spectrum of Activity of Phosphonoacetate and Phosphonoformate

Table 1.6.2 shows the effects of PAA and PFA on a number of polymerases of both viral and cellular origin. The table clearly indicates that PAA and PFA are equally effective as inhibitors of the herpesvirus-induced DNA polymerase. Numerous studies have also revealed that herpesvirus replication in culture is inhibited to the same extent by equimolar concentrations of the two drugs. The two drugs appear to inhibit herpesvirus-induced DNA polymerase by binding to the same site. Evidence for this being derived from detailed kinetic analyses and from studies involving mutants where strains of HSV resistant to PFA have also been found to be resistant to PAA and *vice-versa* (Eriksson and Öberg, 1979; Hay and Subak-Sharpe, 1976; Reno *et al.*, 1978). Cellular DNA polymerase α is also inhibited to the same extent by PAA and PFA, however, the sensitivity of this enzyme to inhibition seems to be related to enzyme concentration

Enzyme	Concentration giving 50 % inhibition, μM	
	PFA(2)	PAA(1)
RNA polymerases		
Influenza virus (Mg^{2+})	20	300
Influenza virus (Mn^{2+})	0.3	0.9
VSV	500	–
Reovirus	>500	–
Calf thymus I	>500	>500
Calf thymus II	>500	>500
E. coli	>500	>500
Reverse transcriptases		
AMV	7	4,000
RMuLV	0.7	600
DNA polymerases		
HSV-1 (strain C 42)	0.4	0.5
HSV-1 (strain 124)	3.5	7.0
Hepatitis B virus	20	>500
Calf thymus α (25 U/assay)	50	75
Calf thymus α (2.5 U/assay)	3.5	6.5
Calf liver γ	>500	>500
Micrococcus luteus	>500	>500
E. coli	>500	>500

Table 1.6.2 Inhibition of polymerases by PAA and PFA (Helgstrand and Oberg., 1980)

(Sabourin *et al.*, 1978) which may explain the earlier conflicting results concerning the sensitivities of various enzyme preparations to PAA (Allaudeen and Bertino, 1978; Bolden *et al.*, 1975; Hay *et al.*, 1977; Mao *et al.*, 1975; Overby *et al.*, 1977).

Table 1.6.2 shows that the antiviral activity of PAA is restricted to members of the herpesvirus group whereas PFA is also an effective inhibitor of influenza RNA polymerase (Helgstrand *et al.*, 1978; Stridh *et al.*, 1979), hepatitis B DNA polymerase (Nordenfelt *et al.*, 1979; Hess *et al.*, 1980), and reverse transcriptase from a number of sources (Sundquist

and Öberg, 1979).

1.6.3 Metabolism and Toxicity of PAA and PFA

Studies utilising radiolabelled-PAA have shown that PAA is not metabolised to other compounds in herpesvirus-infected or uninfected cells (Boezi, 1979) or animals (Bopp *et al.*, 1979). Virtually all of the drug that is administered to rabbits and rodents is rapidly excreted in a chemically unmodified form in the urine and faeces (Bopp *et al.*, 1979; Kung *et al.*, 1978) but a small amount is deposited in the bone (Kung *et al.*, 1978) again as the unmodified form where its turnover appears to be small (Boezi, 1979). PFA has also been shown to accumulate in the bones and cartilage of treated animals (Helgstrand *et al.*, 1979). The effects of PAA and PFA on bone have not been thoroughly evaluated.

Although no systematic investigation into the toxicity properties of PAA and PFA has been undertaken the numerous animal studies reported in the literature contain the salient facts. Fitzwilliam and Griffith (1976) reported that the LD_{50} of PAA for mice was 1500 mg/Kg/day and at concentrations of up to half this figure no gross toxic symptoms were observed. A dose of 150 mg/Kg of PAA was well tolerated when administered intravenously to rabbits but a dose of 300 mg/Kg produced severe muscular spasms and frequently death (Meyer *et al.*, 1976).

Epithelial tissues are irritated by concentrations of PAA above 2% but much higher concentrations of PFA do

not cause irritation. A 2%-5% solution of PAA is well tolerated by rabbits when applied to the eyes but higher concentrations lead to the development of fine punctate lesions of the corneal epithelium which heal within 48 hours of cessation of treatment (Gerstein *et al.*, 1975; Meyer *et al.*, 1976). PAA has been shown to be non-mutagenic in tests using *Salmonella typhimurium* and mouse lymphoma cells (Becker *et al.*, 1976).

1.6.4 Clinical Evaluation

PFA is presently undergoing clinical trials as a topical drug for the treatment of herpesvirus infections. Encouraging results have been obtained with a 3% PFA cream in the treatment of herpes labialis and these studies will be extended to patients with genital herpes.

1.6.5 Mechanism of Action of PAA

Leinbach *et al.* (1976) and Mao and Robishaw (1975) have suggested that PAA inhibits herpesvirus DNA polymerase by interacting with the proposed pyrophosphate binding site of the enzyme. Evidence for this mechanism being derived from several experimental observations: PAA and pyrophosphate are both non-competitive inhibitors of the virus-induced enzyme with respect to the four deoxyribonucleotides and non-competitive inhibitors with respect to DNA when the

deoxyribonucleotide concentration is low but (nearly) uncompetitive inhibitors when the deoxyribonucleotide concentration is high. The apparent inhibition constants being in the mM range for pyrophosphate and 1-2 μM for PAA. PAA is a competitive inhibitor of pyrophosphate in the pyrophosphate-deoxyribonucleoside 5'-triphosphate exchange reaction and in multiple inhibition analyses pyrophosphate and PAA function as mutually exclusive inhibitors, i.e. they both bind to the same site. In addition to these observations, strains of HSV resistant to PAA also show an increased resistance to pyrophosphate (Eriksson and Öberg, 1979; Hay and Subak-Sharpe, 1976; Reno *et al.*, 1978).

The observed inhibition patterns are best explained by an alternate product mechanism as outlined in Fig. 1.6.5 (Leinbach *et al.*, 1976). In the normal polymerisation reaction, pyrophosphate is produced as the nucleoside 5'-triphosphates couple to the growing DNA chain. Pyrophosphate can act as a substrate for the reverse reaction, i.e. pyrophosphate-deoxyribonucleoside 5'-triphosphate exchange. Leinbach *et al.* (1976) propose that PAA, which is a good substrate for the reverse reaction, binds to the enzyme at the pyrophosphate binding site and causes a reversal of the polymerisation reaction. This mechanism predicts that some exchange of pyrophosphate for PAA should take place in the deoxyribonucleoside 5'-triphosphates and that these altered nucleotides should serve as substrates in the polymerisation reaction. This mechanism of inhibition has not been verified by direct experimentation; no altered nucleotides containing

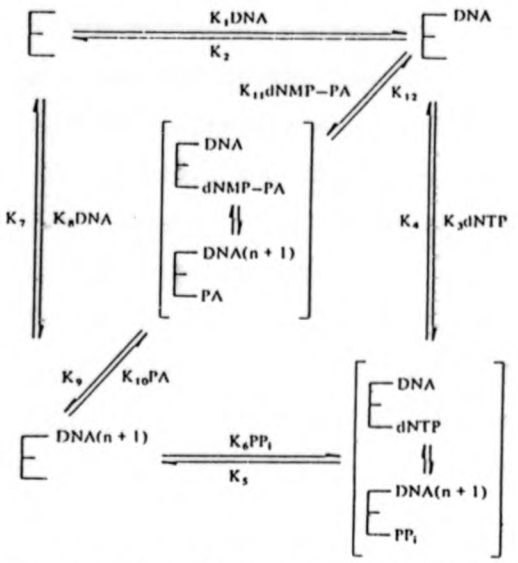


Fig.1.6.5 Proposed mechanism of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase. The basic reaction mechanism in the absence of phosphonoacetate (PA) is a modified ordered bimolecular- bimolecular mechanism. Initial velocity studies and the pyrophosphate (PPi) product inhibition studies presented here are consistent with this mechanism for the herpesvirus-induced DNA polymerase. The postulated compound, dNMP-PA, is a deoxyribonucleoside-5'monophosphate covalently linked to phosphonoacetate by a phosphodiester bond. (LEINBACH et al. 1976)

PAA have been identified in polymerase reaction mixes containing PAA and furthermore deoxyribothymidine 5'-phosphorophosphonoacetate was synthesised and did not act as a substrate for the polymerase (Boezi, 1979 - no experimental details given).

1.7 OUTLINE OF WORK UNDERTAKEN

To date very little has been published on structure-activity relationships of pyrophosphate analogues as inhibitors of influenza RNA polymerase,

and no suggestions as to the molecular mechanism of inhibition have been forwarded. Moreover, the precise mechanism of inhibition of herpesvirus DNA polymerase by analogues of pyrophosphate has not been totally defined with experimental verification even though some ten years have passed since the discovery of the antiviral activity of PAA. Hence, the aim of this project was to study the mechanism of inhibition of polymerases by pyrophosphate analogues.

There would appear to be essentially two possible modes of action of these compounds. Either (a) the compounds are incorporated in altered nucleotides of the type NMP-PAA* which subsequently inhibit the polymerase in a manner analogous to that proposed by Leinbach *et al.* (1976) or (b) they interact directly with the enzyme possibly by coordination to an essential metal ion (possibly zinc). In order to distinguish between these two mechanisms it was necessary to design, synthesise, characterise and screen a range of pyrophosphate analogues for activity against a number of polymerases. Furthermore little has been published on the metal-complexing abilities of pyrophosphate analogues and hence it was also necessary to devise a rapid and efficient method for the determination of pyrophosphate analogue - zinc ion stability constants. It was hoped that elucidation of the precise mechanism of inhibition might lead to the design of more effective inhibitors of viral polymerases.

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*Where N is ribonucleoside or deoxyribonucleoside depending upon the type of polymerase involved.

CHAPTER 2

SYNTHETIC ROUTES

2.1 GENERAL BACKGROUND

Analogues of phosphate esters in which an oxygen atom has been replaced by another atom or functional group have been widely investigated as models which might display modified chemical and biological activity. In the main, attention has been directed to replacement of oxygen by a nitrogen or sulphur atom or a methylene group. Such substitutions can be either 'internal' leading to phosphoramidates (Fig. 2.1 (a) X = NH or NR), thiolophosphates (Fig. 2.1 (a) X = S) and phosphonates (Fig. 2.1 (a) X = CH_2)

Fig. 2.1 Analogues of phosphate esters.

or 'external' forming phosphoramidic acid (Fig. 2.1 (b) Y = NH or NR), phosphorothioates (Fig. 2.1 (b) Y = S) and esters of methylphosphinic acid (Fig. 2.1 (b) Y = CH_2). External substitutions are beyond the scope of this review and will not be discussed further. For further information see Engel (1977) and Blackburn (1981).
The most important difference between the methylene and nitrogen or sulphur containing analogues is the inherent

stability of the P-C bond compared to the P-O, P-N and P-S linkages. Consequently, the search for stable analogues of phosphate esters has focussed largely on phosphonates (Engel, 1977).

2.2 SYNTHESIS OF PHOSPHONATES

2.2.1 Pyrophosphate Analogues

Analogues of pyrophosphate (17) such as PAA (1), PFA (2) and methylenebisphosphonate (8) have been known for many years and can be prepared by either the Michaelis-Becker or Arbuzov reactions (Nylen, 1924; Kossolapoff, 1955; Herrin *et al.*, 1977) (Fig. 2.2.1(i))

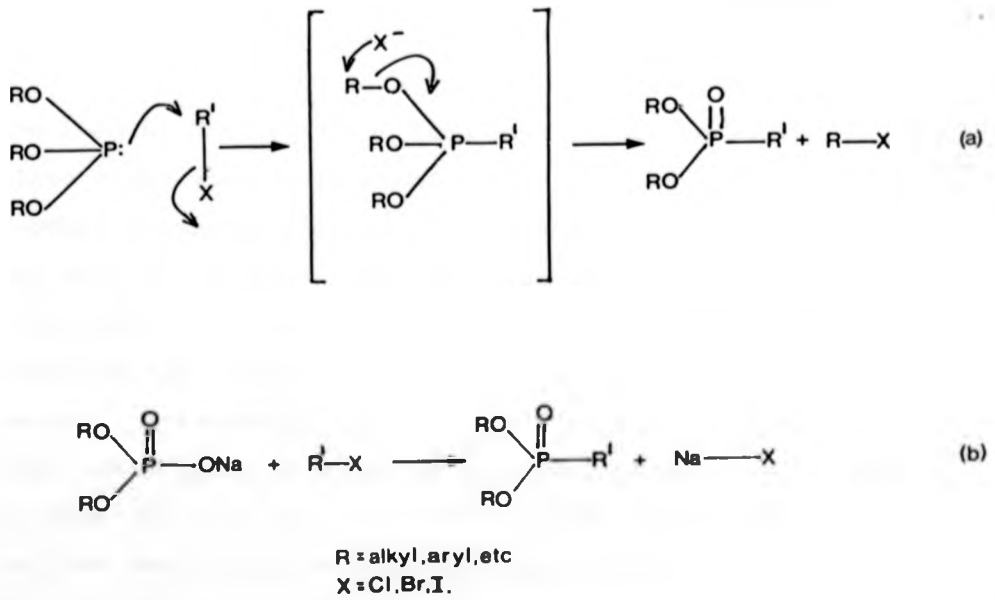

Fig. 2.2.1(i) Arbuzov (a) and Michaelis-Becker (b) reactions.

The final step in these syntheses involves de-esterification to the parent phosphonic acids. Complete de-esterification is usually accomplished under vigorous conditions (typically reflux with concentrated HCl) and often results in low yields. Recently, iodotrimethylsilane (Me_3SiI) has been used to bring about the efficient dealkylation of phosphonate esters (Blackburn and Ingleson, 1980). The dealkylation proceeds in two stages with the initial formation of the silyl ester which is then hydrolysed in water or methanol (Fig. 2.2.1(ii)).

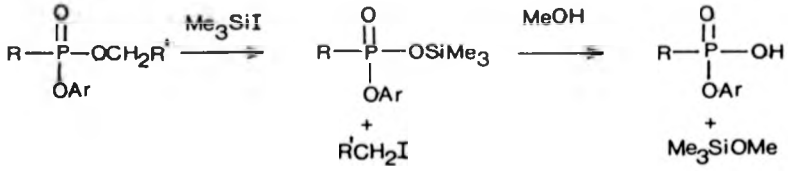

Fig. 2.2.1(ii) Dealkylation of phosphonate esters with Me_3SiI.

The reagent has no effect upon aryl phosphate esters or alkyl carboxylate esters and so can be used to prepare C-ethyl esters of PAA and PFA. Rosenthal *et al.* (1975) and Hata *et al.* (1975) have also reported the use of *tris*(trimethylsilyl)phosphite in Arbuzov reactions to yield readily hydrolysable *bis*(trimethylsilyl)phosphonate esters. P,P-diethyl PAA (7) can be prepared from the fully esterified material by hydrolysis with one equivalent of NaOH (Clayton *et al.* 1979). Thus, derivatives of PAA and PFA modified at either acid function can be readily obtained (Fig. 2.2.1(iii).

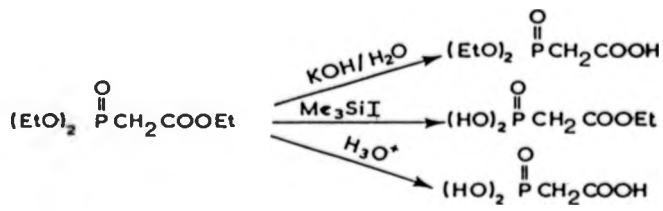

Fig. 2.2.1(iii) Preparation of derivatives of PAA.

The potential stabilisation of a negative charge on a carbon atom with a phosphoryl substituent leads to greatly increased acidity of any hydrogen atom attached to that carbon. Consequently, phosphonates readily form anions on treatment with a variety of bases and these anions will then react with a wide range of electrophiles. Thus, Quimby *et al.* (1968) have described the halogenation of tetraalkyl methylenebisphosphonate with aqueous hypohalite and alkylation via the metalated derivatives.

2.2.2 Nucleoside 5'-Triphosphate Analogues

Analogues of nucleoside 5'-triphosphates in which a methylene group is substituted for the β,γ-pyrophosphate oxygen have been prepared by several methods (Engel, 1977) (Fig. 2.2.2). Carbodiimides have been used successfully but the lack of specificity in effecting condensation between two dissimilar reactants often results in the formation of complex mixtures of products and hence low yields. Mindful of this problem, Chambers and Khorana

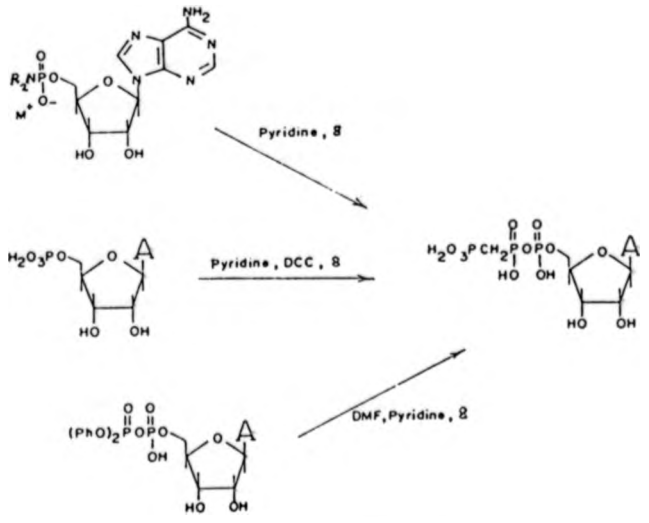

Fig. 2.2.2 Preparation of nucleoside 5'-[β,γ-methylene]triphosphates.

(1958) developed the use of nucleoside 5'-phosphoramidates in which the phosphorus is rendered electrophilic and hence susceptible to attack by a second anion. The use of these activated derivatives effectively eliminates the self condensation of the second anion and other side reactions which are associated with syntheses involving carbodiimides and results in improved yields. The most widely used phosphoramidates are the phosphoromorpholidates which have been chosen because they offer the best compromise between the ease of formation, reactivity and solubility in anhydrous organic solvents (Moffatt and Khorana, 1961).

2.3 BIOLOGICAL APPLICATIONS

With the exceptions of the antiviral properties of PAA (1) and PFA (2), and the use of dichloromethylenebisphosphonate (9) and ethane-1-hydroxy-1,1-bisphosphonic acid (13) in the treatment of certain bone diseases (Fleisch and Felix, 1979), the use of pyrophosphate analogues has been mostly limited to the determination of binding requirements of inorganic pyrophosphatases of diverse biological origin (Kjellstrom and Bishop, 1970; Cooperman and Chin, 1973; Kelly *et al.* 1974). Similarly, the use of nucleoside 5'-[β,γ-methylene]triphosphates has been restricted to mechanistic studies of ATP and GTP dependent processes.

The limited successful application of such compounds as biological models has been attributed in part to the differences in structural characteristics of the P-C grouping compared to the P-O grouping which it replaces (Yount, 1975) (Table 2.3(i)).

Bond	$O(PO_3)_2Na_4(H_2O)_{10}$[a]	$CH_2(PO_3)_2H_4$[b]	$HN(PO_3)_2Na_4(H_2O)_{10}$[c]
P-X	1.612 Å	1.79 Å	1·678 Å
P-O (average)	1.523 Å	1.54 Å	1·521 Å
P-P	2.925 Å	3.05 Å	3·006 Å
P-X-P	130.2°	117°	127°

[a] McDonald and Cruickshank, 1967
[b] Lovell, 1964
[c] Larsen *et al.*, 1969

Table 2.3.(i) Comparison of the geometries of sodium pyrophosphate, methylenebisphosphonic acid and sodium imidobisphosphate (adapted from Yount, 1975).

However, a second and seemingly more important consideration (Blackburn, 1981) is the decrease in acidity of the phosphonic acid groups on introduction of an electron donating alkyl group which results in a different degree of dissociation for the phosphonate compared to the parent phosphate at physiological pH. However, by introducing electron withdrawing groups onto the α-CH_2 group of methylenebisphosphonic acid (8) the similarity of the phosphonate to the parent phosphate can be heightened (Grabenstetter *et al.*, 1967; Blackburn *et al.*, 1981) (Table 2.3.(ii).

Compound $X(PO_3H_2)_2$ where X =	pK_{a4}	pK_{a3}	pK_{a2}
$(CH_3)_2C$	12.10	8.04	3.16
$CH_3C(H)$	11.97	7.49	3.14
CH_2	10.96	7.44	2.87
$CH_3C(OH)$	11.52	7.31	2.89
NH	10.22	7.30	2.60
HC(OH)	10.56	7.05	2.74
BrC(H)	10.15	6.55	2.20
FC(H)	9.35	6.15	< 2.70
Cl_2C	9.78	6.11	-
C=O	8.42	5.81	-
F_2C	8.00	5.80	< 2.60
O	8.22	5.77	2.36

Table 2.3(ii) **Acid dissociation constants of pyrophosphate analogues.**

Table 2.3(ii) clearly shows that there is a progressive increase in acid strength in the series

$$X = CH_2 < CCl_2 < CHF < C(OH)_2 < O \leq CF_2$$

and that difluoromethylenebisphosphonate can essentially be designated an 'isopolar' analogue of pyrophosphate. However, to date little has been reported on the biological activity of halogenated methylenebisphosphates or of nucleotide analogues derived from them.

2.4 MATERIALS AND METHODS

2.4.1 Materials

(i) Unless otherwise stated all compounds were commercially available and were used as received (Appendix III).

(ii) The structures of all the pyrophosphate analogues are shown on page 150.

(iii) All reagents were of analytical grade or were purified before use.

2.4.2 General Methods

(i) ^{1}H n.m.r. spectra were recorded using a Perkin-Elmer R34 spectrometer. ^{31}P n.m.r. spectra were recorded using a Bruker WH90 or WH400 spectrometer and were proton decoupled. ^{31}P n.m.r. chemical shifts are quoted relative to an external standard in p.p.m., downfield shifts are positive.

(ii) Mass spectral analyses are detailed in Appendix I.

(iii) Chromatography was performed by upward development for t.l.c. plates and downward development for paper using the following solvent systems:

System	Plate/Paper	Solvent
I	Whatman 3 MM	EtOH:1 M ammonium acetate (5:2)
II	Whatman 3 MM	MeOH:H_2O:Conc. ammonia (6:1:3)
III	Cellulose F_{254}	Iso-butyric acid:1 M ammonium hydroxide:0.1 M EDTA (100:60:1.6)
IV	Cellulose F_{254}	EtOH:0.5 M ammonium acetate (5:2)

Spots were visualised by fluorescence under 254 nm light in the case of t.l.c. plates. Paper chromatograms were visualised by the method of Mann *et al.* (1979).

(iv) Esters of halogenated methylenebisphosphonates were converted to free acids by boiling with an excess of concentrated hydrochloric acid and unless otherwise stated, were purified as described in the literature.

(v) Interconversion of salts and free acids was accomplished using Dowex 50 ion exchange chromatography.

(vi) Elemental analyses were performed by CHN Laboratories, Leicester, U.K.

2.5 EXPERIMENTAL

2.5.1 Phosphonoacetic Acid (1) was prepared by boiling triethyl phosphonoacetate with an excess of concentrated HCl and was recrystallised from glacial acetic acid. The white crystals were washed with ether and dried in a vacuum oven at 60-70°C overnight. ^{1}H n.m.r. (D_2O, DSS) δ (p.p.m.) 3.0 (d, J_{P-H} 20 Hz, $-CH_2COOH$), ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 16.0, s, M.pt. 136°C (lit. 142°C).

2.5.2 [2-^{3}H]-Phosphonoacetic Acid To anhydrous PAA (5 mg) dissolved in carrier free $^{3}H_2O$ (25 µl) was added concentrated HCl (1 µl) and the mixture heated at 70°C for 5 hours. Excess $^{3}H_2O$ was removed under reduced pressure and exchangeable tritium was removed by repeated addition and evaporation of water. The residue which co-chromatographed on paper with unlabelled PAA in solvent system I was dried *in vacuo* over P_2O_5 to give [2-^{3}H]-phosphonoacetic acid, specific activity 12 µCi/µmole.

2.5.3 P,P-Diethyl Phosphonoacetic Acid (7) was prepared by the method of Clayton *et al.* (1979). Triethyl phosphonoacetate (22.4 g, 0.1 mol) was dissolved in 1 M NaOH (100 ml) and the mixture was stirred for 18 hours at room temperature. The pH was adjusted from 9.5 to 1.5 with dilute HCl, the solution saturated with NaCl and extracted with ethyl acetate (3 x 100 ml). The organic layer was dried and evaporated *in vacuo* to yield

P,P-diethyl phosphonoacetic acid as a liquid (17.8 g, 91%). ^{1}H n.m.r. ($CDCl_3$, TMS) δ (p.p.m.) 9.3 (1H, s, $CO_2\underline{H}$), 4.1 (4H, octet, $CH_3\underline{CH}_2OP$), 2.98 (2H, d, $PC\underline{H}_2CO_2H$), 1.3 (6 H, t, $C\underline{H}_3CH_2$).

2.5.4 Trisodium Phosphonoformate (2) was prepared by the procedure of Nylen (1924) as described by Warren and Williams (1971). Freshly distilled triethyl phosphite (90 g, 0.54 mol) was added to a solution of ethyl chloroformate (54 g, 0.5 mol) in toluene (200 ml). The mixture was heated under reflux for 3 hours and the toluene removed *in vacuo*. The residue was vacuum distilled (142°C/12 mmHg) to yield triethyl phosphonoformate (79 g, 73%). ^{1}H n.m.r. ($CDCl_3$, TMS) δ (p.p.m.) 4.3 (2H, q, $COC\underline{H}_2CH_3$), 4.05 (4H, q, $POC\underline{H}_2CH_3$), 1.33 (3H, t, $COCH_2C\underline{H}_3$) 1.05 (6H, t, $POCH_2C\underline{H}_3$).

To prepare trisodium phosphonoformate, NaOH (10 g, 0.25 mol) was dissolved in water (25 ml) and added dropwise without cooling to triethyl phosphonoformate (10.5 g, 0.05 mol). Heat was evolved and ethanol boiled away. On cooling, needles were obtained which were recrystallised from water to give trisodium phosphonoformate hexahydrate (2.85 g, 19%). ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 1.4, s. Descending paper chromatography, solvent system II, single spot (R_f = 0.59).

2.5.5 C-Ethyl Phosphonoformate (6) Iodotrimethylsilane (3.4 ml, 25 mmol) was added dropwise, over a period of 1 hour to a stirred solution of triethyl phosphonoformate

(2.1 g, 10 mmol) in dry carbontetrachloride (10 ml) at 25°C. On completion of addition the mixture was stirred for a further 1 hour. The solution was extracted with water (2 x 10 ml) and the pooled aqueous extracts were lyophilised. C-Ethyl phosphonoformate was obtained as a colourless oil (1.51 g, 98%). ^{1}H n.m.r. (D_2O, HOD) δ (p.p.m.) 4.05 (2H, q, $COC\underline{H}_2CH_3$), 3.05 (3H, t, $COCH_2C\underline{H}_3$). ^{31}P n.m.r. (d_5-pyridine, 85% H_3PO_4) δ (p.p.m.) 28.2, s.

2.5.6 2-Phosphonopropionate (3) was isolated as its monocyclohexylammonium salt following the acid hydrolysis of the corresponding triethyl ester and was recrystallised from aqueous ethanol. Anal. for $C_9H_{20}O_5NP$, calcd. (found), C 42.74 (42.85), H 7.79 (8.13), N 5.47 (5.55). ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 17.35, s. M.pt. 212-213°C.

2.5.7 Methylenebisphosphonic Acid (8) was prepared by acid hydrolysis of the corresponding tetraisopropyl ester. The crude product was repeatedly evaporated to dryness from *iso*-propanol and dried in a desiccator over P_2O_5. ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 18.2, s. M.pt. 200-201°C.

2.5.8 Tetraisopropyl Dichloromethylenebisphosphonate was prepared essentially as described by Quimby *et al.* (1968). Tetraisopropyl methylenebisphosphonate (6.37 g, 19 mmol) was added with vigorous stirring to a solution (140 ml) containing NaOCl (5%) and NaCl (4%). The mixture was stirred for 30 minutes on ice and then for a further

30 minutes at room temperature. The resulting white solid was removed by suction filtration and the aqueous solution was extracted with chloroform (3 x 20 ml). The white solid was dissolved in the chloroform extracts and the solution was extracted with water until a negative halide test was observed. The chloroform was removed under reduced pressure and the residue distilled. White crystals of tetraisopropyl dichloromethylenebisphosphonate were obtained upon standing. ^{31}P n.m.r. ($CDCl_3$, 85% H_3PO_4) δ (p.p.m.) 6.86, s. Free acid (9) ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 7.46, s.

2.5.9 Tetraisopropyl Dibromomethylenebisphosphonate

Tetraisopropyl methylenebisphosphonate (25 g, 0.072 mol) was added to a solution of potassium carbonate (250 g, 55%). Bromine (3.84 ml, 0.144 mol) in heptane (20 ml) was added with vigorous stirring over a period of 2 hours with the temperature maintained at 40°C. Water (100 ml) was added and the two layers were separated. The aqueous layer was extracted with chloroform (100 ml) and discarded. The organic portions were combined, extracted with water (2 x 100 ml) and dried. The organic solvents were removed *in vacuo* and a ^{31}P n.m.r. assay of the residue revealed 76% dibromo-, 16% monobromo- and 8% methylenebisphosphonate. The residue was recycled through the above procedure to produce tetraisopropyl dibromomethylenebisphosphonate, 96% pure by ^{31}P n.m.r. ($CDCl_3$, 85% H_3PO_4) δ (p.p.m.) 6.5, s. Free acid (11) ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 6.5, s.

2.5.10 Tetraisopropyl Monohalomethylenebisphosphonates (10 and 12) were prepared from the corresponding dihalo-derivatives by the same general procedure (Nicholson and Vaughn, 1971). The preparation of monochloromethylene-bisphosphonate is described in detail below.

A solution of sodium hydrosulphide (2.8 g, 0.05 mol) in water (20 ml) was slowly added to tetraisopropyl dichloromethylenebisphosphonate (20.3 g, 0.05 mol) in methanol (20 ml). The temperature being maintained at 25°C throughout the addition. The solution was stirred for 30 minutes and filtered to remove sulphur. The filtrate was extracted with chloroform (50 ml) and the organic portion dried. Removal of solvent left a colourless oil. Vacuum distillation (165°C/2 mmHg) gave tetraisopropyl monochloromethylenebisphosphonate. ^{31}P ($CDCl_3$, 85% H_3PO_4) δ (p.p.m.) 11.5, s. Free acid (10), ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 11.3, s. Tetraisopropyl dibromomethylenebisphosphonate ^{31}P n.m.r. ($CDCl_3$, 85% H_3PO_4) δ (p.p.m.) 6.4, s. Free acid (12) ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 6.5, s.

2.5.11 Methanehydroxybisphosphonate (14) was prepared according to Quimby *et al.* (1967). Tetrasodium mono-halomethylenebisphosphonate was refluxed with 1 M NaOH for approximately 8 hours. The resulting solution was titrated with dilute HCl to pH 5 and disodium methane-hydroxybisphosphonate was precipitated in 73% yield by the addition of methanol. ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 15, s.

2.5.12 Carbonylbisphosphonate (15) was prepared as described by Quimby *et al.* (1967). Dichloromethylene-bisphosphonic acid (1.27 g, 5.2 mmol) was heated under reflux with NaOH (3.1 g, 78 mmol) in water (30 ml) for 1½ hours. Addition of methanol crystallised out crude, hydrated tetrasodium carbonylbisphosphonate which was recrystallised from aqueous acetonitrile (2.52 g, 88%). ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 0.0, s. U.V. λmax, 419 nm. I.R., C=O str, 1612 cm^{-1}.

2.5.13 Dimethylaminomethylenebisphosphonate (19) The free acid obtained from the acid hydrolysis of the corresponding tetraethyl ester (8.7 g, 26 mmol) was recrystallised from methanol to yield the desired product as a white crystalline powder (2.85 g, 50%). ^1H n.m.r. (D_2O, DSS) δ (p.p.m.) 3.16 (6H, s, $N(C\underline{H}_3)_2$), 3.72 (1H, t, $\underline{H}C\text{-}N(CH_3)_2$).

2.5.14 Nucleoside 5'-triphosphate analogues (20, 21, 23 and 24) were prepared by the same general procedure which was essentially that of Moffatt and Khorana (1961). The synthesis of adenosine 5'-phosphorophosphonoacetate (AMP-PAA, 20) is considered typical and is described in detail below.

4-Morpholine-N,N'-dicyclohexylcarboxamidinium adenosine 5'-phosphoromorpholidate (710 mg, 1 mmol) and PAA (1.4 g, 10 mmol) were dried by repeated evaporations from anhydrous pyridine. The two reactants were mixed as solutions in pyridine and co-evaporated (< 40°C). The residue was taken up in pyridine (50 ml) and shaken at

room temperature, the reaction being followed by t.l.c. in solvent systems III and IV (and also by ^{31}P n.m.r., see Section 4.2.7). On completion of reaction (1-2 hours), the pyridine was removed *in vacuo*, the residue taken up in water (200 ml) and applied to a DEAE DE52 column (CH_3COO^- form, 2 x 45 cm). The column was washed extensively with water and then eluted with a linear gradient of triethylammonium acetate (0.1 to 0.5 M, pH 4.7, 3L). The eluate was monitored by u.v. absorption at 254 nm and the required fractions, as determined by t.l.c., were pooled and evaporated to dryness *in vacuo* (< 40°C). The residue was evaporated from water (x 6) to remove triethylammonium acetate, converted to the sodium salt, purified by gel filtration, lyophilised, and stored desiccated at -20°C.

Anal. for $C_{12}H_{14}O_{11}N_5Na_3P_2$ calcd. (found), C 26.93 (26.02), H 2.64 (2.67), N 13.09 (12.19), P 11.57 (11.94). ^{31}P n.m.r. (D_2O, 85% H_3PO_4) δ (p.p.m.) 5.2 (d, J_{p-p} 26.4 Hz, P^{β}), -9.7 (d, J_{p-p} 25 Hz, P^{α}). T.l.c. (III, R_f = 0.4; IV, R_f = 0.07). pH 1.0, λ_{max} 260 nm (ε 14500); pH 7.0, λ_{max} 257 nm (ε 14300); pH 12.3, λ_{max} 259 nm (ε 16000).

Analyses for the other nucleoside triphosphate analogues synthesised are given in Table 2.5.14.

	Compound	^{31}P n.m.r. δ (p.p.m.)			t.l.c. R_f		
		P^{α}	P^{β}	P^{γ}	III	IV	λ_{max} nm (pH 7.0)
21	AMP-PCP	-10.83 (d)	14.27 (q)	11.34 (d)	0.4	0.05	257
23	AMP-$PCCl_2P$	-10.20 (d)	2.63 (q)	8.71 (d)	0.35	0.05	257
24	dAMP-PAA	-10.86 (broad)	10.59 (d)	-	0.38	0.07	259

Table 2.5.14 Analyses for nucleoside triphosphate analogues.
^{31}P n.m.r. chemical shifts are quoted relative to external 85% H_3PO_4.

CHAPTER 3

HERPESVIRUSES

3.1 METHODS

3.1.1 Cell Culture and Media

HEp-2 cells were used for the preparation of viral DNA polymerases. Serially propagated African green monkey kidney cells (vero) were used for infectivity assays. HEp-2 cells were grown as monolayer cultures in roller bottles (2.5 L) at 37°C under overlaying medium (∿ 150 ml) of HEPES-buffered Glasgow modification of Eagle's medium (GMEM) supplemented with non-essential amino acids (NEAA, 1%), newborn calf serum (NCS, 10%), L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). For maintenance of cells the concentration of NCS was reduced to 1%. Vero cells were grown as monolayer cultures in flat culture bottles (175 ml) at 37°C under overlaying medium (100 ml) of HEPES-buffered Dulbecco's modification of Eagle's medium supplemented with NCS (10%), L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). For maintenance of cells the concentration of NCS was reduced to 1%. In all media the concentration of sodium bicarbonate was adjusted to meet the required buffering capacity. Cells were passaged according to conventional procedures using a trypsin (0.05%), EDTA (0.02%)

HEPES-buffered balanced salt solution. Viable cell counts were determined by means of trypan blue dye exclusion.

3.1.2 Preparation of Virus Stocks

The HFEM strain of HSV-1 and the 3345 strain of HSV-2 were employed. The PAA resistant strain (P^r) was HSV-1 18 Clone I (Honess and Watson, 1977) originally supplied to Roche Products by R. Honness, N.I.M.R., Mill Hill, London. HSV stocks were crude suspensions prepared from confluent monolayers of HEp-2 cells in roller bottles which were infected at a multiplicity of infection (m.o.i.) of 0.001 plaque forming units (p.f.u.) per cell in maintenance media (10 ml). This low m.o.i. was employed to avoid the appearance of defective virus (Watson *et al.*, 1966). After absorption (2 hours/37^{o}C), virus inocula were removed and fresh, warm maintenance media (50 ml) was added. The infected cells were incubated for 3-4 days until cytopathic effect (c.p.e.) was clearly visible. The cells were scraped into the media using a rubber policeman and pelleted by low speed centrifugation (2000 rpm/15 minutes/4^{o}C). The cells were then resuspended in spent culture medium (1 roller bottle/5 ml) and sonicated (full power/1 minute, 0 to 4^{o}C, MSE soniprobe) to release virus. The solution was clarified by centrifugation (2000 rpm/15 minutes/4^{o}C). The supernatant was aliquoted, stored at -70^{o}C and thawed once prior to use.

3.1.3 Plaque Reduction Assay

A confluent 175 cm^3 flat culture bottle of vero cells was trypsinised. The detached cells were suspended in growth medium (9 ml) and a viable cell count was carried out. The cells were diluted to 2×10^5 cells/ml in growth medium and 24 well plates (Falcon or Costar) were inoculated with cell suspension (1 ml). After 24 hours at 37°C the cells were confluent and the growth medium was removed. Each well was infected with freshly diluted virus in maintenance medium (50 pfu in 0.2 ml). After absorption (1 hour/4°C) overlay (0.7 ml of maintenance medium containing 1% carboxymethyl cellulose) was added to each well. To this was added the various drug dilutions (0.1 ml) in duplicate wells. After incubation for 3-4 days at 37°C in a humidified atmosphere of carbon dioxide (5%) in air, the monolayers were fixed with formal saline solution (∿ 3 ml, 4% (v/v) formaldehyde in phosphate buffered saline (PBS)/well/15 minutes). The formal saline was removed and Giemsa was added as stain. The plates were washed with a constant stream of water and dried. Viral plaques were counted under a dissection microscope and the average number at each concentration of test compound was calculated as a percentage of that obtained for the drug free control. The drug concentration required to reduce the control figure by 50% (ID_{50}) was calculated by interpolation from a semi-logarithmic plot. Cytoxicity was observed as change in the microscopic appearance of the cells.

3.1.4 Induction of DNA Polymerase Activity in HSV-Infected Cells

The procedure employed was essentially that of Powell and Purifoy (1977). Confluent monolayers of HEp-2 cells in roller bottles (approx. 10^8 cells/roller bottle) were infected with HSV at a m.o.i. of 2-10 pfu/cell in maintenance medium (10 ml), or mock infected, and incubated (1 hour/37°C). At the end of the absorption period virus and mock inocula were removed and fresh warm maintenance medium (50 ml) was added. The cells were incubated until c.p.e. was just apparent (10-18 hours/37°C). Monolayers were then washed with cold PBS (3 x 50 ml) and the cells were scraped into a small volume of cold PBS using a rubber policeman and pelleted by low speed centrifugation (2000 rpm/5 minutes/4°C). Enzyme extracts were prepared immediately or the washed cell pellets were stored at -70°C and thawed once prior to use. Cell pellets stored in this manner retain DNA polymerase activity for several months (D. Purifoy, personal communication). Enzyme extracts were prepared as follows, all procedures being carried out at 0-4°C; cells were resuspended in buffer (10 mM *tris*-HCl, pH 7.5, 3 mM 2-mercaptoethanol) at a concentration of 10^7 cells/ml. The cells were then sonicated (1 minute/full power, MSE soniprobe). An equal volume of high salt buffer was added to give final concentrations of 1.7 M NaCl and 5 mM EDTA and the mixture was allowed to stand for 40 minutes. DNA and protein precipitates were removed by centrifugation (30,000 xg/20 minutes). The supernatant

was dialysed overnight against buffer (2 x 3 L, 10 mM *tris*-HCl, pH 7.5, 3 mM 2-mercaptoethanol, 10% (v/v) glycerol). The dialysate was clarified by centrifugation (100,000 xg/60 minutes), stored in aliquots at -70°C and thawed once prior to use.

3.1.5 Preparation of Activated DNA Template

A modification of the procedure of Schlabach *et al.*, (1971) was employed. Native salmon sperm DNA (2.5 mg/ml in 10 mM *tris*-HCl, pH 7.4, 5 mM $MgCl_2$) was incubated with pancreatic DNase I (40 ng/mg DNA) at 60°C for 5 minutes. Phenol (5 mg) was then added to the DNA solution to inactivate the DNase and the solution was dialysed overnight at 4°C against buffer (2 x 3 L, 10 mM *tris*-HCl, pH 7.4) to eliminate magnesium ions, small nucleotides and phenol. The dialysate was divided, stored at -20°C and thawed once prior to use.

3.1.6 DNA Polymerase Assays

The standard assay for HSV-induced DNA polymerase contained in a final volume of 200 µl: 50 mM *tris*-HCl, pH 7.8, 5 mM $MgCl_2$, 20 mM KCl, 110 mM $(NH_4)_2SO_4$, 200 µM EDTA, 6 mM 2-mercaptoethanol, 25 µM each of dATP, dCTP, dGTP, 6 µM [methyl-^{3}H]-TTP (1000-2000 cpm/pmol), 375 µg/ml DNase I treated salmon sperm DNA and enzyme. 50 µl of enzyme extract incorporated about 20 pmol dTMP per 200 µl reaction mixture in 30 minutes.

The standard assay for calf thymus DNA polymerase α contained in a final volume of 200 µl: 50 mM *tris*-HCl, pH 7.8, 1 mM dithiothreitol, 500 µg/ml BSA, 10 mM $MgCl_2$, 20 mM KCl, 200 µg/ml DNase I treated salmon sperm DNA, 20 µM [^{3}H-]dTTP (sp. act 400-1000 cpm/pmol) 100 µM each of dATP, dCTP, dGTP and enzyme (0.01 unit). One unit incorporated 10 nmol of dTMP into acid precipitable material in 60 minutes under the conditions employed.

Reaction mixes were maintained at 0°C until zero time. The reactions were initiated by the addition of enzyme and the tubes were incubated at 37°C for 30 minutes in the case of HSV-induced DNA polymerase and for 60 minutes for calf thymus DNA polymerase α, during which time incorporation of radionuclide into acid-precipitable material increased linearly. Reactions were stopped by the addition of cold TCA (200 µl, 20% (w/v)) and the mixtures were kept on ice for at least 2 hours. Acid-insoluble material was collected by suction filtration onto GF/C discs (Whatman) wetted with 10% (w/v) TCA. Each tube was rinsed with 10 ml of cold 10% (w/v) TCA. Discs were washed once with cold 10% TCA, twice with 5% TCA, once with ethanol and then oven dried at 80°C. Radioactivity was measured by scintillation counting in a toluene based scintillant. In the case of HSV-induced DNA polymerase assays the counts for the mock infected system for each assay were subtracted from those of the virus system. All tests were run in duplicate. Unless otherwise stated, test compounds were added to reaction mixes before the addition of virus. 50% Inhibition values were obtained from the dose-

response curve for each compound.

3.2 EXPERIMENTAL AND RESULTS

3.2.1 Effect of Pyrophosphate Analogues on HSV-Induced DNA Polymerases

Table 3.2.1 lists the pyrophosphate analogues examined and summarises their corresponding inhibition of isolated HSV-induced DNA polymerase of both type 1 and type 2. The table shows a systematic variation in structure in which the bridging oxygen of pyrophosphate has been replaced with a methylene group or omitted, as in the case of PFA and phosphonoformamide(5), and the acid groups are either phosphono or carboxyl. PAA gave 50% inhibition of HSV-induced DNA polymerase at concentrations of 2 and 7 μM for type 1 and type 2 respectively. Shortening the distance between the two acid groups as in PFA produced an equally effective inhibitor. However, increasing the carbon chain length (3-phosphopropionic acid (4)) or substitution on the methylene group (2-phosphonopropionic acid (3)) resulted in a decrease in activity.

Of the compounds containing two phosphono groups linked by a group other than oxygen only carbonylbisphosphonate (15) produced any inhibition, causing a 50% reduction in type 1 and type 2 polymerase activity at 180 μM.

Amongst the nucleoside triphosphate analogues

Table 3.2.1 Effects of Pyrophosphate Analogues on Isolated HSV DNA Polymerases and in Plaque Reduction Assays

Compound	Concn. Producing 50% Inhibition[a]			
	HSV-1 (HFEM)		HSV-2 (3345)	
	Plaque Redn. (µg/ml)	DNA Pol. (µM)	Plaque Redn. (µg/ml)	DNA Pol. (µM)
1	30 (215 µM)	2	40 (285 µM)	7
2	85 (280 µM)	3	50 (185 µM)	4
3	> 500	150	> 500	150
4	> 500	> 500	> 500	> 500
5	> 500	> 500	> 500	> 500
6	> 500	> 500	> 500	> 500
7	> 500	> 500	> 500	> 500
8	> 500	> 500	> 500	> 500
9	> 500	> 500	> 500	> 500
10	> 500	> 500	> 500	> 500
11	> 500	> 500	> 500	> 500
12	> 500	> 500	> 500	> 500
13	> 500	> 500	> 500	> 500
14	40% inhibn. at 50 µg/ml	> 500	> 500	> 500
15	> 500[b]	180	> 500[b]	180
16	> 500	> 500	> 500	> 500
17	> 500	> 500	> 500	> 500
18	> 500	> 500	> 500	> 500
20	190 (300 µM)	300	115 (190 µM)	180
19	> 500	> 500	> 500	> 500
21	> 500	> 500	> 500	> 500
22	> 500	> 500	> 500	> 500
23	> 500	> 500	> 500	> 500
24	> 500	> 500	> 500	> 500

[a]Calculated from dose-response curve for each compound.

[b]Observable cytoxic effect at 500 µg/ml.

examined only AMP-PAA (20) was found to possess any activity causing a reduction of 50% in type 1 and type 2 polymerase activity at 190 μM and 300 μM respectively.

3.2.2 Effect of Pyrophosphate Analogues on HSV Plaque Formation

Both PAA and PFA reduced plaque formation of HSV type 1 and type 2 by 50% at a concentration of 50 μg/ml (200-300 μM). Methanehydroxybisphosphonate (14) also affected plaque formation to a slight extent at 500 μg/ml. AMP-PAA was effective in culture producing a 50% reduction of type 1 plaque formation at 190 μg/ml (300 μM) and type 2 plaque formation at 115 μg/ml (190 μM). None of the other compounds studied produced any significant inhibition of plaque formation at 500 μg/ml (Table 3.2.2).

3.2.3 Effect of Pyrophosphate Analogues on a PAA Resistant (P^r) Strain of HSV-1

Fig. 3.2.3 shows the dose-response curves obtained for PAA against the activity of DNA polymerases derived from cells infected with P^s strains of HSV types 1 and 2 (HFEM and 3345 respectively) and a P^r strain of HSV type 1 (18 Clone I). The diagram shows that the DNA polymerase derived from the P^r strain is approximately 100 fold more resistant to PAA than the parent P^s strain. Table 3.2.3 summarises the effect of some pyrophosphate analogues on the induced DNA

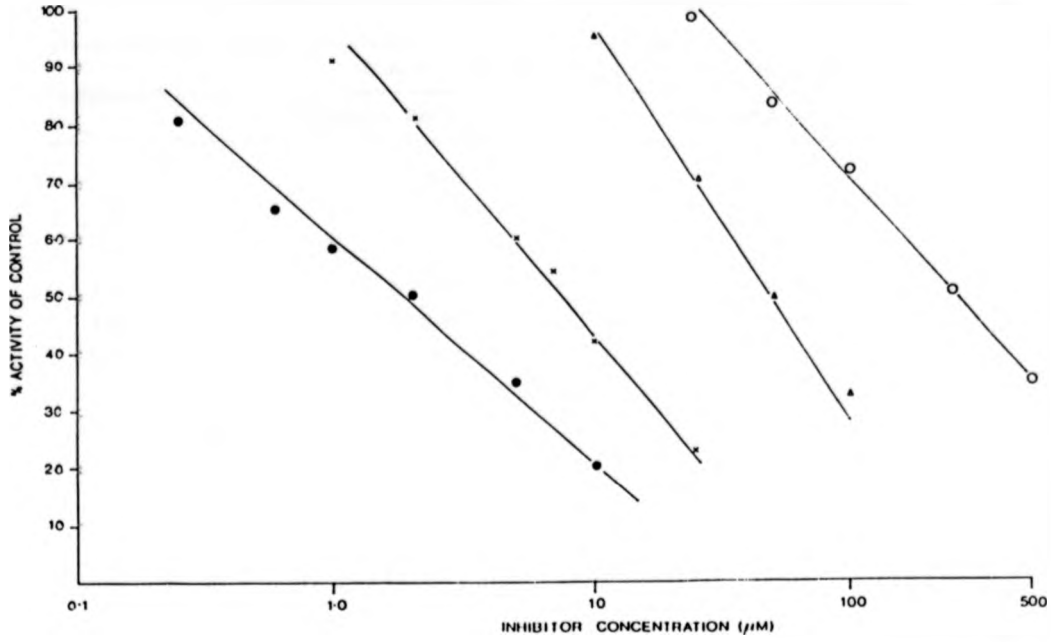

Fig. 3.2.3 Specificity of PAA to various DNA polymerases. DNA polymerases were assayed as described under methods. The inhibition is expressed as the percentage of uninhibited DNA polymerase activity remaining at various concentrations of inhibitor. The symbols used are: HSV-1 (HFEM) (●); HSV-2 (3345) (X); Calf thymus DNA polymerase α (Δ) and HSV-1 (18 Clone I) (O).

polymerase and on the formation of plaques in culture.

3.2.4 Effect of Pyrophosphate Analogues on Calf Thymus DNA Polymerase α

Table 3.2.4 lists the effects of pyrophosphate analogues on calf thymus DNA polymerase α. Only

Table 3.2.3 Effect of some Pyrophosphate Analogues on HSV-1 18 Clone I (P^r) Isolated DNA Polymerase and in Plaque Reduction Assays

Compound	Concn. Producing 50% Inhibition[a]	
	Plaque Redn. (μg/ml)	DNA Pol. (μM)
1	> 500	250
2	> 500	250
3	> 500	> 500
14	> 500	> 500
15	> 500[b]	> 500
20	> 500	> 500

[a]Calculated from relevant close-response curves.
[b]Observable cytoxic effect at 500 μg/ml

Table 3.2.4 Effect of Pyrophosphate Analogues on Calf Thymus DNA Polymerase α[a]

Compound	Concn. Producing 50% Inhibition (μM)
1	45
2	50
11	350
15	100
23	350

[a]All other compounds examined were inactive at a concentration of 500 μM.

one of the compounds active against HSV-induced DNA polymerase was more active against calf thymus DNA polymerase α, namely carbonylbisphosphonate producing 50% inhibition at 100 μM. AMP-PAA had no effect on the cellular enzyme at 500 μM. PAA inhibited HSV type 1 DNA polymerase by 50% at a concentration of 2 μM whereas 50 μM was needed to inhibit calf thymus DNA polymerase α to a similar extent. Two compounds which produced no inhibition of HSV-induced DNA polymerases at 500 μM were however active against the cellular enzyme. Dibromomethylenebisphosphonate (11) and AMP-PC(Cl)$_2$P (23) produced 50% inhibition at 450 μM and 350 μM respectively.

3.2.5 Effect of Substrate Concentration on Inhibition of HSV-1 (HFEM) DNA Polymerase by PAA and AMP-PAA

To characterise the nature of inhibition further the effect of substrate concentration on PAA and AMP-PAA inhibition was studied. In the experiment the four deoxyribonucleoside triphosphates were used as the variable substrates and activated DNA was at saturating concentration (375 μg/ml). When the data was plotted employing the method of Lineweaver and Burk (1934) (Fig. 3.2.5), straight lines could be drawn intersecting in the lower left hand quadrant which, using the nomenclature of Cleland (1963), is described as linear non-competitive inhibition. The k_i's for PAA and AMP-PAA were calculated as 6 and 360 x 10^{-6} M respectively.

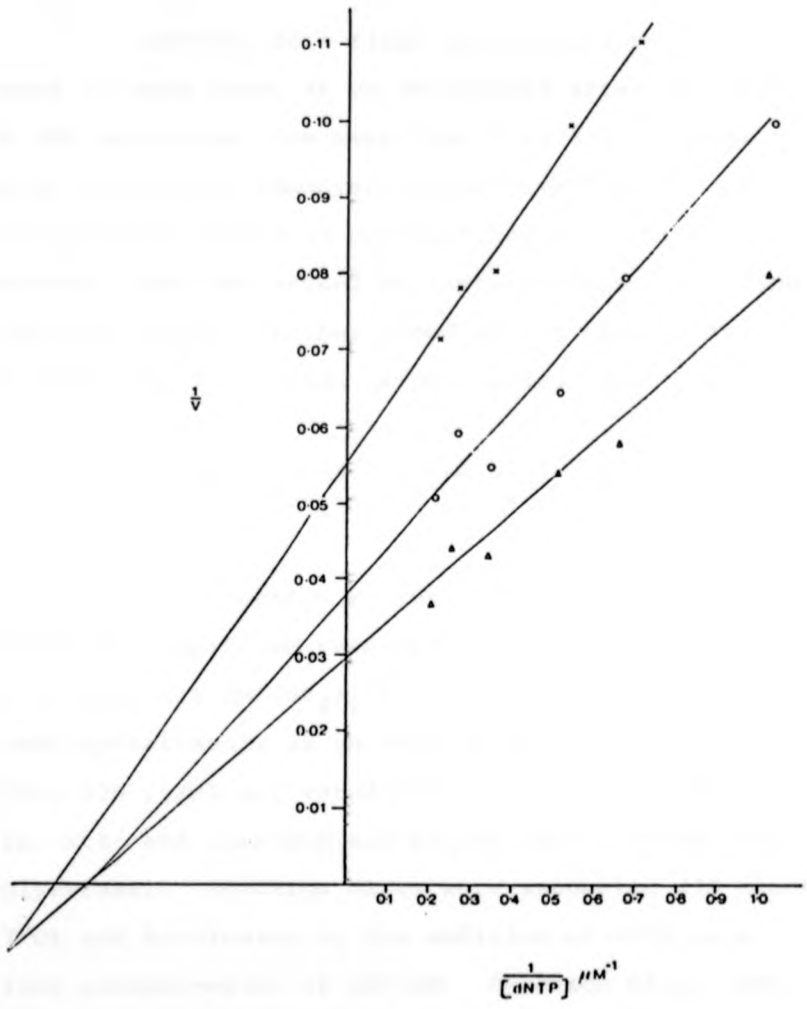

Fig. 3.2.5 Effect of PAA and AMP-PAA on the reaction rate of HSV-1 (HFEM) DNA polymerase in the presence of varying concentrations of dNTP's. Assay conditions were essentially as outlined in Methods. The symbols used are: PAA, 2 μM (O); AMP-PAA, 500 μM (X); control, no drug, (Δ).

3.2.6 Time-Course of AMP-PAA Inhibition

AMP-PAA to a final concentration of 500 μM was added at zero time, 15 or 30 minutes after the initiation of DNA synthesis. As seen from Fig. 3.2.6, under the assay conditions employed radioactive substrate was incorporated into acid-precipitable material for at least 90 minutes. AMP-PAA caused an instantaneous inhibition of identical extent whether added at the time of initiation or after the initiation of polymerisation.

3.2.7 Generation of PAA Containing Nucleotides in Polymerisation Reactions

The reaction mixture contained in a final volume of 1 ml:50 mM *tris*-HCl, pH 7.8, 5 mM $MgCl_2$, 20 mM KCl, 110 mM $(NH_4)_2SO_4$, 200 μM EDTA, 6 mM 2-mercaptoethanol, 25 μM each of dATP, dCTP, dGTP and dTTP, 375 μg/ml activated DNA, 20 μM [2-^{3}H}-PAA (sp. act. 600 cpm/pmol) and enzyme (HSV-1 (HFEM) DNA polymerase). Reaction mixes were incubated (30 minutes/ 37^{o}C) and terminated by the addition of EDTA to a final concentration of 200 mM. Reaction mixes were applied to a column of DEAE DE52 cellulose (0.6 x 20 cm, $CH_3.COO^-$ form). The column was washed extensively with water and eluted with a linear gradient (100 ml) of 0 to 0.5 M triethylammonium acetate, pH 4.7. The flow rate was adjusted to approximately 0.2 ml/minute and fractions (2 ml) were collected. The radioactivity

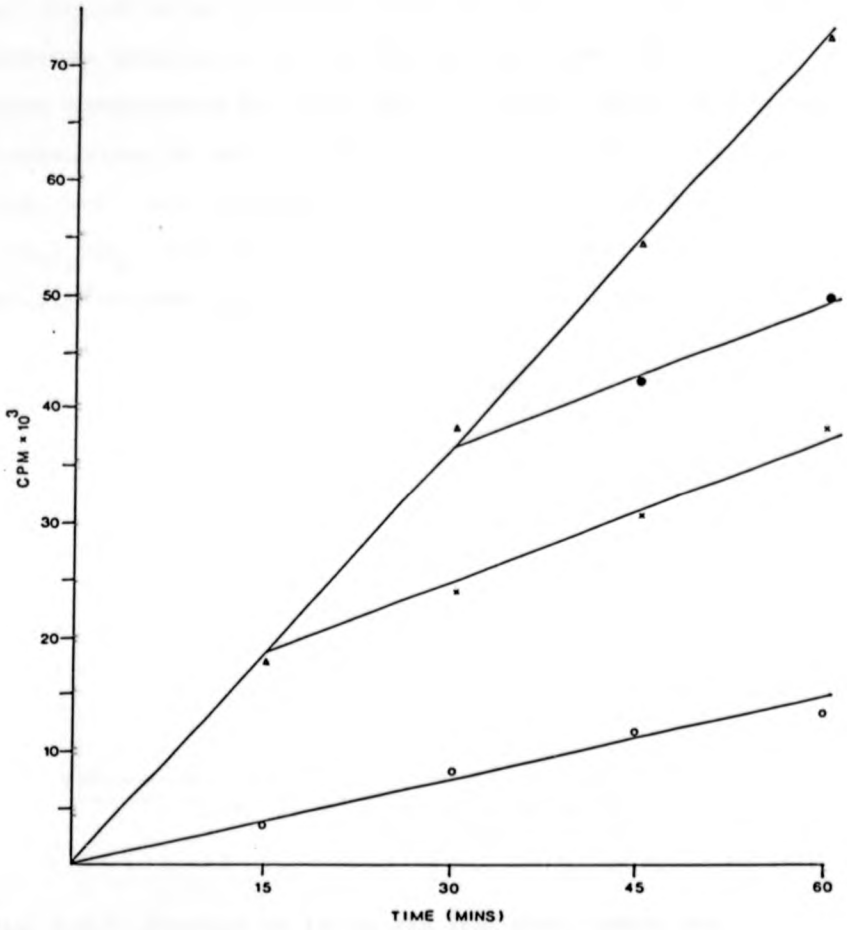

Fig. 3.2.6 Time-course of inhibition of HSV-1 (HFEM) DNA polymerase by AMP-PAA. The reaction conditions were as described under Methods. AMP-PAA (20) at a final concentration of 500 μM was added at 0(O), 15 (X) or 30 (●) minutes after the initiation of polymerisation. A reaction mixture without the addition of AMP-PAA was used as control (Δ).

of aliquots (100 µl) of each fraction was determined by liquid scintillation counting (Fig. 3.2.7). The elution positions of [2-^{3}H]-PAA and dAMP-PAA (24) were determined by DEAE DE52 chromatography of a sample containing 50 mM *tris*-HCl, pH 7.8, 20 µM [2-^{3}H]-PAA (sp. act. 600 cpm/pmol), 5 mM $MgCl_2$, 20 mM KCl, 110 mM $(NH_4)_2SO_4$, 200 mM EDTA and 5 mM dAMP-PAA. Recoveries of [2-^{3}H]-PAA from the column were at least 85%.

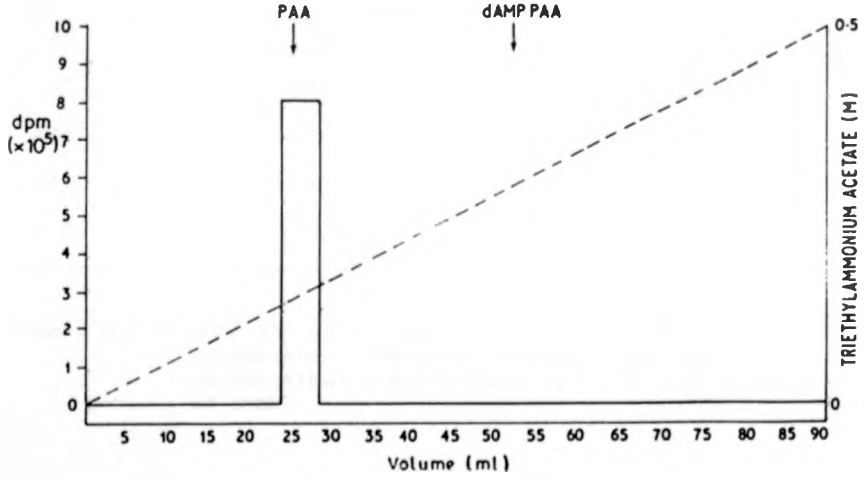

Fig. 3.2.7 Recovery of [2-^{3}H]-PAA from HSV-1 (HFEM) DNA polymerisation reactions. Experimental details are described in the text.

3.2.8 dAMP-PAA As A Substrate for HSV-1 (HFEM) DNA Polymerase

It was shown that dAMP-PAA could not serve as a substrate in place of dATP in the HSV-1 (HFEM) DNA polymerase assay as follows: The enzyme was assayed in

standard reaction mixtures in the absence of dATP, in the presence of increasing amounts of dAMP-PAA and in the presence of saturating amounts of dATP (Table 3.2.8).

Addition (μM)		Cpm incorporated into acid-precipitable material
dAMP-PAA	dATP	
0	0	1510
5	0	1606
10	0	1451
25	0	1490
50	0	1570
100	0	1503
500	0	1485
0	100	38245

Table 3.2.8 dAMP-PAA as a substrate for HSV-1 (HFEM) DNA polymerase. The assay procedure was essentially as described under Methods but with the omission of dATP.

3.3 DISCUSSION

The initial problem encountered in this study was the choice of test system. A number of contradictory reports have appeared in the literature concerning changes in the sensitivity of HSV DNA polymerases to PFA during the course of their purification (Ostrander and Cheng, 1980; Derse *et al.*, 1982). Rationalisation of these observations is made difficult by the differences in biological origin of the polymerases

and the experimental conditions employed. It can be argued that whereas a crude extract of infected cells may contain components which might interfere with the assay procedures, the purification procedure may remove some components which confer sensitivity to an inhibitor. The ease of preparation coupled to the availability of a number of specific controls, in particular the well characterised inhibitors PAA and PFA and PAA resistant (P^r) strains of HSV, led to the choice of crude extracts of HSV-infected cells as test systems.

Infection of HEp-2 cells with HSV leads to the induction of a new DNA polymerase activity which increases linearly for at least 12 hours and remains at this elevated level for at least a further 12 hours (Purifoy and Benyesh-Melnick, 1975). Monolayer cultures of HEp-2 cells were infected at a m.o.i. of 2-10 pfu/cell to ensure a synchronous infection of as large a number of cells as possible. Cells were harvested at approximately 16 hours post-infection and the crude suspensions obtained by sonic oscillation of cell pellets were extracted using the high salt procedure of Powell and Purifoy (1977) which solubilises proteins bound to DNA and removes the bulk of DNA from solution. This latter point was confirmed by the finding that the activity of HSV DNA polymerase preparations was dependent upon exogenous template. The crude extracts so obtained were assayed under high-salt conditions which stimulated the HSV DNA polymerase and produced almost total inhibition of cellular DNA

polymerases, which is in agreement with previous work (Weissbach *et al.*, 1973). The high ionic strength of the assay procedure is thought to mimic the action of polyamines in infected cells which activates HSV DNA polymerase and inhibits the HSV (exo-endo)DNase (Ostrander and Cheng, 1980) which co-purifies with HSV DNA polymerase (Knopf, 1979; Ostrander and Cheng, 1980). It should be noted at this point that storage of the extract at -70°C for 7-10 days results in total abolition of the viral DNase activity (Grossberger and Clough, 1981; R. Honness personal communication). Under conditions optimal for cellular DNA polymerases all extracts derived from both infected and mock-infected cells were shown to possess some activity indicating that cellular enzymes were extracted along with viral polymerases.

The DNA template used in the various assays was an 'activated' DNA prepared by treatment of salmon sperm DNA with bovine pancreatic DNase I. The procedure employed for its preparation was essentially that of Schlabach *et al.* (1971) except that inactivation of DNase I was achieved by the addition of phenol rather than by heating which was found to be not fully effective. DNA treated in this manner contains an optimal number of 3'-OH groups and single stranded gaps which permit repair synthesis. It is pertinent to note that maximal levels of HSV DNA polymerase activity are not obtained in infected cells until replication of viral DNA is essentially over suggesting that HSV DNA polymerase may also play a role in recombination and/or repair (Powell and Benyesh-Melnick,

1975).

The simple combination of a phosphono and a carboxyl group either linked directly (PFA), or joined by a methylene group (PAA), produced the most effective inhibitors in both the cell-free and cell culture systems. Esterification of either the phosphono or the carboxyl groups, increase in carbon chain length or substitution on the methylene group of PAA led to a decrease in, or abolition of activity. These findings being in good agreement with a number of previous studies (Eriksson *et al.*, 1980; Leinbach *et al.*, 1976). The nucleotide analogue AMP-PAA was found to possess slight inhibitory activity in both the cell-free and cell culture systems. The activity of this compound against HSV DNA polymerase would appear not to be a result of breakdown to release free PAA as ^{31}P n.m.r. and t.l.c. studies have shown the material to be stable under the assay conditions employed. However, the stability of AMP-PAA under the conditions employed in the cell culture model is unknown. Hence metabolism or simple degradation resulting in the generation of free PAA should also be considered as a possible route to inhibition in this case.

Analogues of methylenebisphosphonate which contain electron withdrawing groups on the methylene bridge have been found to be effective inhibitors of influenza RNA polymerase (Chapter 4). However, of such compounds examined, only carbonylbisphosphonate had any inhibitory effect upon HSV DNA polymerase and this compound was ineffective in cell culture. The

inhibition of plaque formation by methanehydroxybisphosphonate did not correspond to its effects upon HSV DNA polymerase. It seems likely that the activity of methanehydroxybisphosphonate in the cell culture model is a result of the cell-toxic effects of this compound (Stenberg, 1981). It would appear therefore that the structural requirements for inhibitors of HSV DNA polymerase are more specific than those for inhibitors of influenza RNA polymerase which is presumably a manifestation of differences in the respective pyrophosphate binding sites.

The HSV DNA polymerase appears to be more sensitive to PAA, PFA and AMP-PAA than the cellular DNA polymerase α. However, carbonylbisphosphonate was a slightly more effective inhibitor of the cellular enzyme than the viral enzyme. The inhibition profiles obtained for PAA, PFA and carbonylbisphosphonate are in agreement with reports by Eriksson *et al.* (1980) on calf thymus DNA polymerase α and by Allaudeen and Bertino (1978) on DNA polymerase α from a murine lymphoid leukaemia cell line. Dibromomethylenebisphosphonate and AMP-PC(Cl)$_2$P have not previously been reported as being inhibitory towards calf thymus DNA polymerase α. Further studies on their mode of inhibition have yet to be undertaken. It is pertinent to point out that biological activity cannot be attributed to the removal of free metal ions (Mg^{2+}, K^{+}) from solution by chelation as the compounds are active at concentrations which would not significantly affect the free metal ion concentration.

Compounds active against the DNA polymerase derived from cells infected with a strain of HSV-1 (HFEM) known to be sensitive to PAA (P^S) were also screened for activity against the DNA polymerase derived from cells infected with a PAA resistant (P^r) strain of HSV-1, 18 Clone I. This polymerase was approximately 100 fold more resistant to each compound compared to the 'parent' P^S strain, suggesting that these compounds all bind to the same site on the polymerase. No compound produced any significant reduction in plaque number at 500 µg/ml. Further evidence supporting the view that AMP-PAA and PAA bind to the same site on the polymerase comes from kinetic studies with HSV-1 (HFEM) DNA polymerase. Both compounds gave linear non-competitive inhibition when the dNTP's were present as the variable substrates. The results obtained with PAA are consistent with a number of previous reports (Mao and Robishaw, 1975; Leinbach *et al.*, 1976). The time-course of inhibition for AMP-PAA was also essentially similar to that reported by Mao and Robishaw (1975) for PAA.

The alternate product inhibitor mechanism proposed by Leinbach *et al.* (1976) to explain the inhibition of herpesvirus of turkeys DNA polymerase by PAA, predicts that altered nucleotides of the type dNMP-PAA would be formed in DNA polymerisation reactions containing PAA. Experiments designed to test the predictions of this hypothesis were performed but no supporting evidence was obtained. No altered nucleotides containing [2-^{3}H]-PAA were recovered from DNA polymerisation reactions containing [2-^{3}H]-PAA

and furthermore, the nucleotide analogue dAMP-PAA was prepared and was found to be neither a substrate for nor an inhibitor of HSV DNA polymerase. Similar results but with no accompanying experimental details have been reported by Boezi (1979) using dTMP-PAA.

CHAPTER 4

INFLUENZA VIRUS

4.1 METHODS

4.1.1 Preparation of Virus Stocks

The influenza virus A/X49 was a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. The virus was grown in the allantoic sacs of fertile hens eggs and was isolated essentially as described by Kelly and Dimmock (1974). Eleven day old embryonated hens eggs (5 dozen) were inoculated with infected allantoic fluid (0.1 ml of a 10^{-3} dilution in phosphate buffered saline, PBS). The eggs were incubated (33°C/48 hours) and then chilled (-20°C/2 hours). The allantoic fluid was collected, carefully excluding blood and yolk, and centrifuged (3000 rpm/20 minutes) to remove unwanted egg membranes. From this point onwards all procedures were carried out at 0 to 4°C. The supernatant was removed and the virus was pelleted by centrifugation (21,000 rpm/90 minutes, 6 x 250 ml rotor). The supernatant was discarded and the virus pellet was allowed to soak overnight in PBS. The pellet was then resuspended in PBS and layered onto a velocity gradient of 10 to 40% (w/v) sucrose in buffer (30 ml, 10 mM *tris*-HCl, pH 7.4) and centrifuged (22,000 rpm/1 hour, 3 x 65 ml swing-out rotor).

The diffuse virus band was collected by bottom puncture and the sucrose was diluted out with PBS to a final volume of 30 ml. The virus suspension was then layered onto an equilibrium gradient of 30 to 70% (w/v) sucrose in buffer (30 ml, 10 mM *tris*-HCl, pH 7.4) and centrifuged (20,000 rpm/overnight, 3 x 65 ml swing-out rotor). The virus band was collected, diluted with PBS and the virus pelleted by centrifugation (30,000 rpm/ 2 hours, 8 x 50 ml rotor). The supernatant was discarded and the pellet was allowed to soak overnight in PBS. The virus was then resuspended in buffer (3 ml, 400 µM *tris*-HCl, pH 8.0) and frozen as aliquots at -70°C and thawed once prior to use.

4.1.2 Haemagglutination Assay

Serial two-fold dilutions of the virus preparation were made in PBS. Chick red blood cells (0.5 ml, 0.5% suspension) were added to each virus dilution (0.5 ml) and the test mixtures were incubated (room temperature/1 hour). The pattern formed by the red blood cells was noted. The last well giving definite agglutination was considered the end point which is expressed as the reciprocal of that dilution.

4.1.3 Influenza RNA Dependent RNA Polymerase Assay

The standard reaction mixture contained in a final volume of 200 µl: 50 mM *tris*-HCl, pH 8.0, 5 mM

magnesium acetate, 150 mM potassium chloride, 5 mM dithiothreitol (DTT), 0.4 mM adenylyl-(3'-5')-guanosine, 0.25% Nonidet P-40, 0.4 mM each of ATP, GTP, CTP, 0.005 mM [^{3}H]UTP (5 µCi) and purified virus. Reaction mixes were maintained at 4^{o}C until zero time. The reaction was initiated by addition of virus and the tubes were incubated at 30^{o}C for 1 hour. The amount of virus added was such that the incorporation of [^{3}H]UTP into acid precipitable product increased linearly during the incubation period. The reaction was stopped by the addition of ice-cold saturated sodium pyrophosphate (200 µl) followed by cold 10% (w/v) TCA (2 ml). The tubes were vortexed and kept on ice for 15 minutes or longer. The precipitates were collected by suction filtration onto Whatman GF/C discs, which were washed several times with 10% TCA, once with ethanol and dried. The radioactivity of material precipitated on the discs was then determined by scintillation counting using a toluene-based scintillant. Pyrophosphate analogues were added to the reaction mixtures before addition of virus. The concentration of analogue which inhibited the incorporation of [^{3}H]-uridine into acid-insoluble material by 50%, was derived from the dose-response curve for each compound.

4.1.4 Determination of Pyrophosphate Analogue - Zinc Ion Stability Constants

The stability constants were determined by

the method of Hummel and Dreyer (1962) using a column of Sephadex G-10 (1.6 x 92 cm) which had been equilibrated with zinc chloride (10 μM, Spectroscopic grade) in buffer (0.1 M triethanolamine-HCl, pH 8.0). The pyrophosphate analogues (100 nmol) were added in buffer (1 ml, triethanolamine/zinc) and the column eluted with the same buffer at a flow rate of 0.4 ml/minutes. The zinc content of individual fractions (2 ml) was determined by atomic absorption spectrometry and the stability constants determined as described by Hummel and Dreyer (1962).

A detailed description of this technique can be found in Appendix II which is a reprint of N. Yoza, *J. Chem. Ed.*, 54, 247 (1977).

4.2 EXPERIMENTAL AND RESULTS

4.2.1 Dose-Response Curves

A typical dose-response curve is shown in Fig. 4.2.1. The strongest inhibitor amongst the compounds examined was dibromomethylenebisphosphonate (11) which produced 50% inhibition of influenza RNA polymerase at a concentration of 10 μM (Table 4.2.1). All halo-substituted methylenebisphosphonates, (9) to (12), were potent inhibitors whereas methylenebisphosphonate (8) was ineffective. Replacement of the bridging oxygen of pyrophosphate with an imido (16) and a carbonyl (15) function also produced effective inhibitors. Of the compounds containing a

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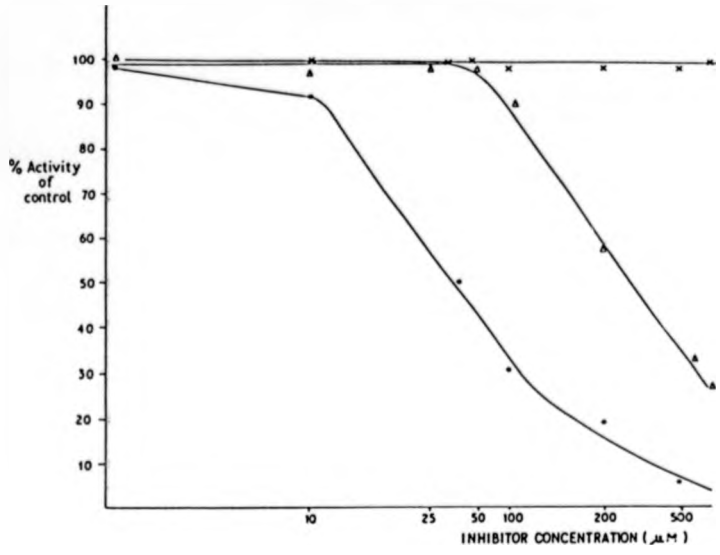


Fig. 4.2.1 Effect of pyrophosphate analogues on influenza RNA polymerase. Influenza RNA polymerase was assayed as described in Methods. The inhibition is expressed as the percentage of the uninhibited RNA polymerase activity remaining at various concentrations of inhibitor. The symbols used are AMP-PAA(X), PAA(Δ) and PFA(●). In the uninhibited reaction ∿ 7500 cpm were incorporated.

phosphono and a carboxyl function, only PFA was an effective inhibitor. Increasing the distance between the two acid groups by the introduction of a methylene group, (PAA), caused a decrease in potency and this slight activity was destroyed by a further increase in the length of the carbon chain (3-phosphonopropionic acid, 4) or by substitution on the methylene group (2-phosphonopropionic acid, 3). Furthermore, esterification of either the carboxyl (6) or phosphono (7)

Table 4.2.1 Inhibitory effect of Pyrophosphate Analogues on RNA Polymerase from Influenza Virus and Calf Thymus DNA Polymerase α

Compound	$pK_{d'}$*	Concn. (μM) Producing 50% Inhibition	
		Influenza RNA Polymerase	Calf Thymus DNA Polymerase α
1	5.5	275	45
2	5.6	35	35
3	~5	> 500	> 500
4	< 4	> 500	> 500
5	< 4	> 500	> 500
6	< 4	> 500	> 500
7	< 4	> 500	> 500
8	5.3	> 500	> 500
9	> 6	75	> 500
10	> 6	85	> 500
11	> 6	10	350
12	> 6	45	> 500
13	> 6	> 500	> 500
14	5.2	350	> 500
15	5.4	20	100
16	5.7	50	> 500
17	5.7	125	> 500
18	> 6	> 500	> 500
19	ND	> 500	> 500
20	ND	> 500	> 500
21	ND	> 500	> 500
22	ND	> 500	> 500
23	ND	> 500	> 350
24	ND	> 500	> 500

*Dissociation constant of complex formed with zinc ions, measured at pH 8.0 as described in text.

ND Not determined

groups of PAA also destroyed activity.

4.2.2 Time-Course of Inhibition by PFA

PFA (500 μM) was added at zero time, 15 and 30 minutes after the initiation of polynucleotide synthesis. As seen from Fig. 4.2.2 the radioactive substrate was incorporated into polynucleotide at a linear rate for at least 60 minutes. Addition of PFA caused an instantaneous inhibition of identical extent whether added at the time of initiation or after the initiation of polymerisation.

4.2.3 Effect of Zinc Ions and a Reducing Agent (DTT) on Inhibition by PFA

In order to determine if inhibition of influenza RNA polymerase by PFA was caused by interaction of the inhibitor with zinc ions or sulphydryl groups present on the enzyme, assays were performed in the presence and absence of zinc ions, DTT and PFA (Table 4.2.3).

Addition	[^{3}H]-UMP Incorporated into Acid-Precipitable Material	
	Control*	+ 100 μM PFA
None	2925	571
5 mM DTT	4741	1445
100 μM Zn^{2+}	185	0
100 μM Zn PFA	851	-

Table 4.2.3 Effects of zinc ions and DTT on inhibition by PFA

*Assay procedures were essentially as described under methods but with the omission of DTT.

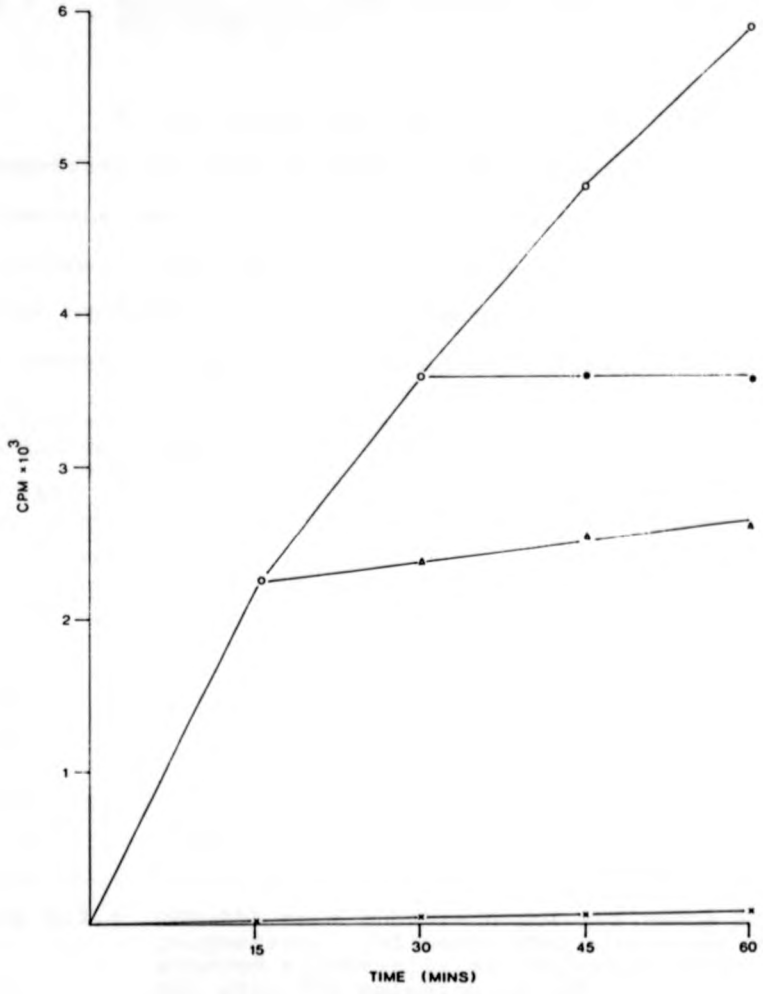


Fig. 4.2.2 Time-course of inhibition by PFA. Assay conditions were as described in Methods. PFA at a concentration of 500 µM was added at zero time (X), and 15 (Δ) and 30 minutes (●) after the initiation of polymerisation. A reaction mixture without the addition of PFA was used as control (O).

4.2.4 AMP-PAA as a Substrate for the Influenza RNA Polymerase

It was shown that AMP-PAA could not serve as a substrate in place of ATP in the influenza RNA polymerase assay as follows: The enzyme was assayed in standard reaction mixtures in the absence of ATP, in the presence of increasing amounts of AMP-PAA and in the presence of saturating amounts of ATP (Table 4.2.4).

Addition (μM)		cpm Incorporated into Acid-Precipitable Material
AMP-PAA	ATP	
0	0	463
50	0	1034
100	0	598
250	0	281
500	0	840
1000	0	836
0	400	7507

Table 4.2.4 AMP-PAA as a substrate for influenza RNA polymerase. Influenza RNA polymerase was assayed essentially as described in Methods but with the omission of ATP.

Similar results were obtained for the ATP analogue AMP-PC(Cl)$_2$P.

4.2.5 Recovery of [2-^{3}H]-Phosphonoacetate from Influenza RNA Polymerase Assays

To the standard assay mixture (1 ml), containing

0.4 mM UTP in place of [^{3}H]-UTP, was added [2-^{3}H]-PAA (0.5 μmole) and purified virus. The reaction was incubated at 30°C for 1 hour then EDTA was added to a final concentration of 20 mM. The reaction mixture was applied to a DEAE DE52 column ($CH_3.COO^-$ form, 0.6 x 14 cm) which was washed with water and then eluted with a linear gradient of triethylammonium acetate (0.5 M, pH 4.7, 100 ml). The radioactivity of the fractions (1.5 ml) was then determined by liquid scintillation counting in a toluene based scintillant. The recovery of radioactivity from this experiment was almost 90%. The relative elution positions of [^{3}H]-PAA and AMP-PAA were determined in a separate experiment (Fig. 4.2.5).

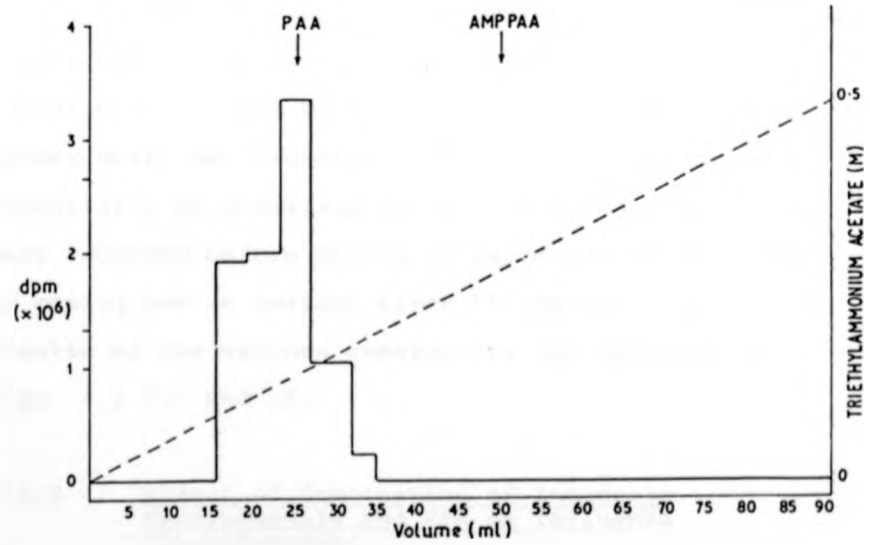


Fig. 4.2.5 Recovery of radioactivity from influenza RNA polymerase assays containing [2-^{3}H]-PAA. Reaction conditions were as outlined in the text.

4.2.6 Effect of Substrate Concentration on Inhibition of Influenza RNA Polymerase by PFA

The mechanism of inhibition of influenza RNA polymerase by PFA was studied by varying the concentration of substrate nucleoside triphosphates in the polymerase assay system. However, when the data obtained were transferred into double reciprocal plots according to the procedure of Lineweaver and Burk (1934), the lines obtained were curved and no conclusions could be made concerning the type of inhibition.

4.2.7 Preparation of ATP Analogues followed by ^{31}P n.m.r.

The reaction of adenosine 5'-phosphoromorpholidate with pyrophosphate analogues in pyridine (3 ml, 10 mM tubes) was followed by ^{31}P n.m.r. at 162 MHz using a Bruker WH400 spectrometer. Reaction conditions were essentially as described in Section 2.5.14. Spectra were recorded before mixing of reactants at the time of mixing and at various times thereafter. The results of the various experiments are depicted in Figs. 4.2.7.i and ii.

4.2.8 Effect of Combination of Inorganic Pyrophosphate and PFA on Influenza RNA Polymerase

To determine if inorganic pyrophosphate displayed a competitive or additive effect with PFA,

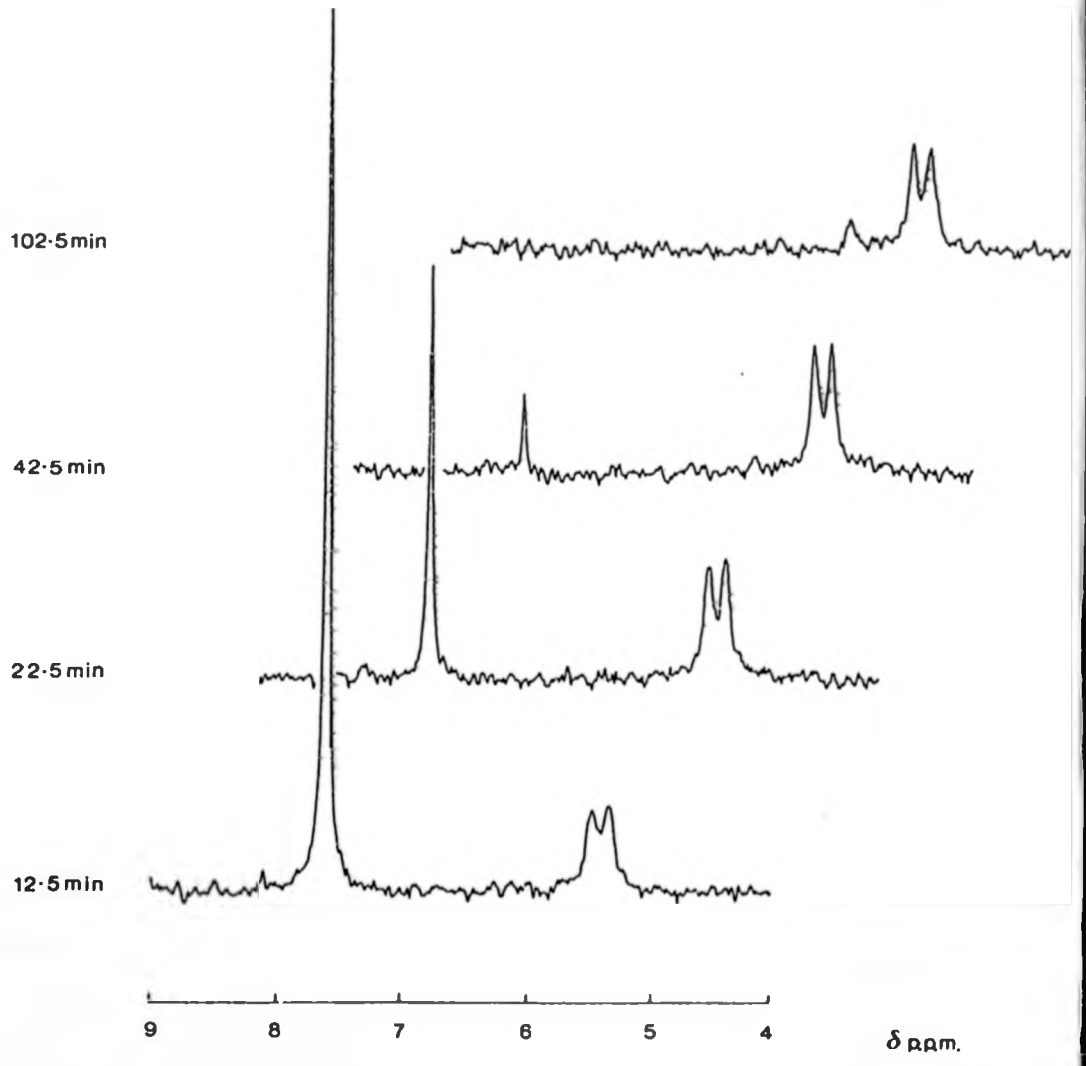


Fig. 4.2.7.i Adenosine 5'-phosphoromorpholidate plus phosphonoacetic acid in pyridine, partial ^{31}P n.m.r. spectra after various times.

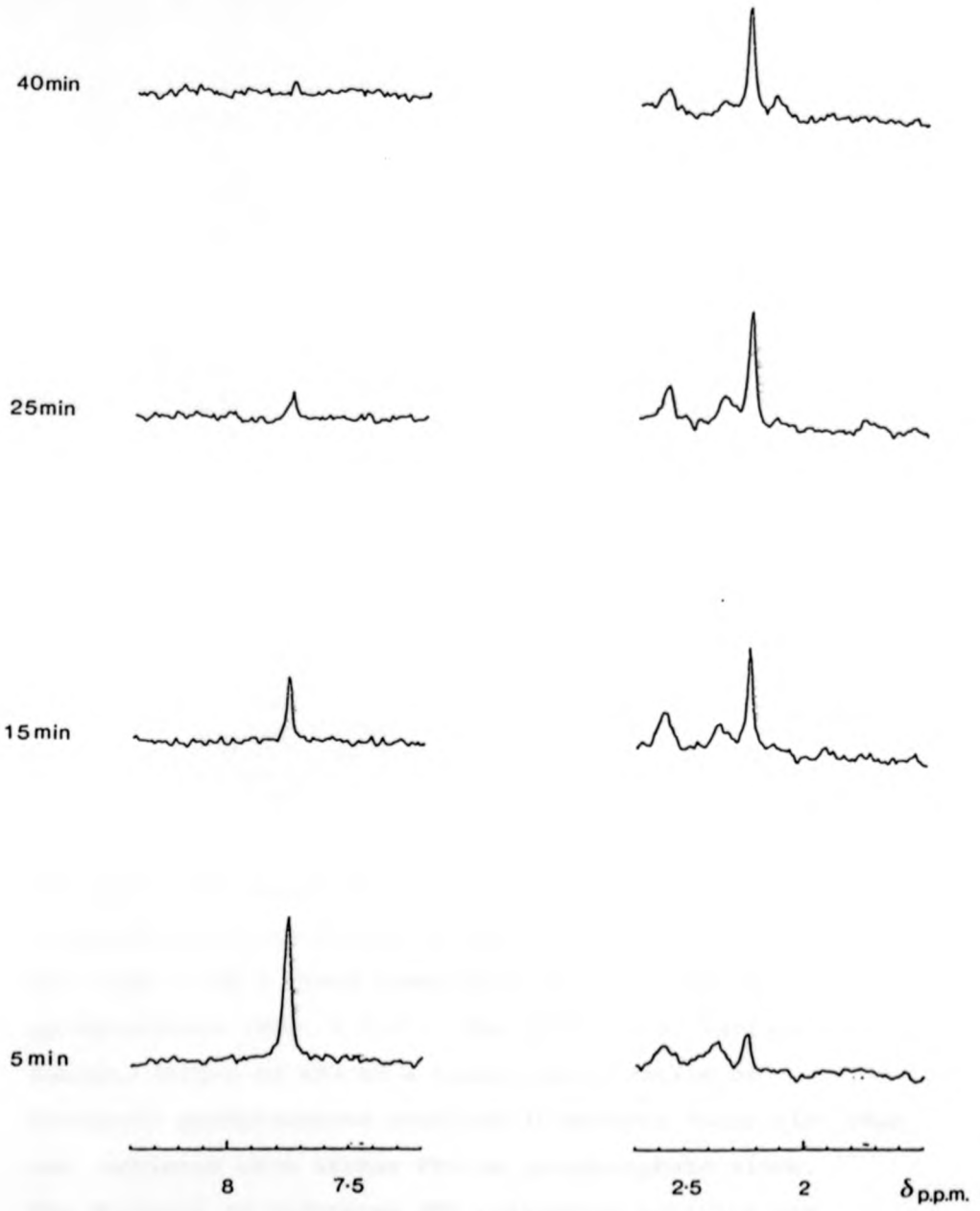


Fig. 4.2.7.ii Adenosine 5'-phosphoromorpholidate plus phosphonoformic acid in pyridine. ^{31}P n.m.r. spectra taken at various times after subtraction of T = 0 spectrum.

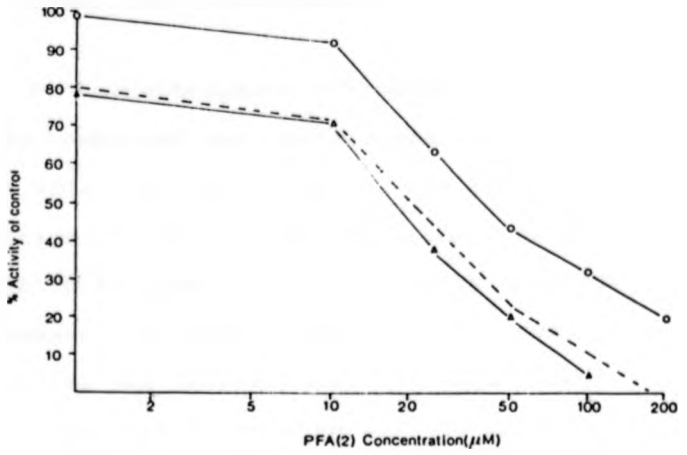


Fig. 4.2.8 Effect of combination of PFA and inorganic pyrophosphate on influenza RNA polymerase activity. Assay conditions were as described in Methods. The inhibition is expressed as percentage of uninhibited activity remaining at various concentrations of inhibitor. Additions: control, no addition (O), inorganic pyrophosphate, 50 μM (Δ). Dotted line represents calculated plot for an additive effect.

influenza RNA polymerase was assayed in the presence of increasing concentrations of PFA both in the presence and absence of a fixed concentration of inorganic pyrophosphate (Fig. 4.2.8). The addition of various concentrations of PFA to a fixed concentration of inorganic pyrophosphate resulted in greater inhibition than was achieved with either PFA or pyrophosphate alone. The decrease in influenza RNA polymerase activity was not significantly different from that calculated for an additive effect.

4.2.9 Effect of Pyrophosphate Analogues on free Metal Ion Concentration

The pyrophosphate analogues in this study are acidic compounds and could conceivably exert their antiviral effect by chelation to, and hence removal from solution of, metal ions (Mg^{2+} ions) which have been shown to be essential for activity of influenza RNA polymerase. However, compounds were active at μM levels whereas the concentrations of Mg^{2+} and K^{+} ions present in the assays was 5 mM and 150 mM respectively. Under such conditions no change in the level of free metal ion concentration would be expected and hence biological activity cannot be attributed to depletion of free metal ions.

4.3 DISCUSSION

Influenza RNA polymerase was assayed in detergent-disrupted virus preparations using endogenous RNA as template and in the presence of adenylyl-(3'-5')-guanosine (ApG) as primer, which has been shown to stimulate polymerase activity *in vitro* (Plotch and Krug, 1977). This system was chosen as it is not yet possible to isolate active influenza RNA polymerase which is free from RNA and other proteins. Recently, a ribonucleoprotein consisting of PA, PB_1 and PB_2 and associated with vRNA but free of other proteins has been isolated which is capable of transcribing vRNA *in vitro* (Kawakami and Ishihama, 1983). However, the total characterisation of the processes carried out by this complex remains to be

published.

The inhibition of the polymerase was followed by studying the effect of pyrophosphate analogues on the incorporation of radioactivity into acid-insoluble polynucleotide. The assay is technically simple but stringent precautions must be taken to prevent contamination of reagents with RNase which would result in virus preparations appearing to have little or no RNA polymerase activity. Hence, all heat stable reagents were autoclaved prior to use and an aseptic technique was employed.

There are essentially two possible mechanisms for the mode of action of the pyrophosphate analogues. Either (a) they are converted by the virus into analogues of nucleoside triphosphates which inhibit the influenza RNA polymerase, or (b) they interact directly with the polymerase, possibly by coordinating with an essential metal ion, possibly zinc.

Results suggest that these inhibitors do not act by being incorporated into the β-γ positions of nucleoside triphosphate analogues. The ATP analogue AMP-PAA (20) is neither a substrate for, nor an inhibitor of influenza RNA polymerase. Dichloromethylenebisphosphonic acid (9) is an effective inhibitor of the viral polymerase but its ATP analogue (23) is again neither a substrate for, nor an inhibitor of this enzyme. Furthermore, when [2-^{3}H]-phosphonoacetate was included in the standard polymerase assay mixture, all the radioactivity recovered was in the form of starting material. None could be detected in the form of nucleoside triphosphate analogues.

The ATP analogue AMP-PAA is easy to prepare by the phosphoromorpholidate route. However, attempts to prepare the ATP analogue which contains PFA at the β-γ position met with failure. All that was achieved was the rapid conversion of adenosine 5'-phosphoromorpholidate into AMP. To gain a greater insight into the course of these conversions the reactions were followed by ^{31}P n.m.r. With PAA there was steady decay of the phosphoromorpholidate signal at 7.8 ppm accompanied by an increase in intensity of a pair of doublets centred at 5.2 and -9.7 ppm due to the formation of the pyrophosphate residue in AMP-PAA. When PFA is used the phosphoromorpholidate signal decays as before but no doublets appear, only a singlet at 2.2 ppm due to AMP. When the C-ethyl ester of PFA, which is not an inhibitor of influenza RNA polymerase, is used, signals due to the phosphoromorpholidate at 7.8 ppm and the ester at 28.2 ppm are observed at the start of the experiment and with time, two doublets of equal intensity appear at 20.2 ppm and -9.4 ppm due to the formation of the ATP analogue.

It seems likely that PFA reacts with the phosphoromorpholidate to give an ATP analogue but that it rearranges rapidly to give a highly reactive mixed anhydride of adenosine 5'-phosphoric and formic acids which subsequently breaks down (Fig. 4.3.i) (Jaenicke and Koch, 1963). With PAA the nucleoside triphosphate analogue could still decompose by an intramolecular mechanism but here a six-membered rather than a five-membered ring would be involved and hence this breakdown should be comparatively

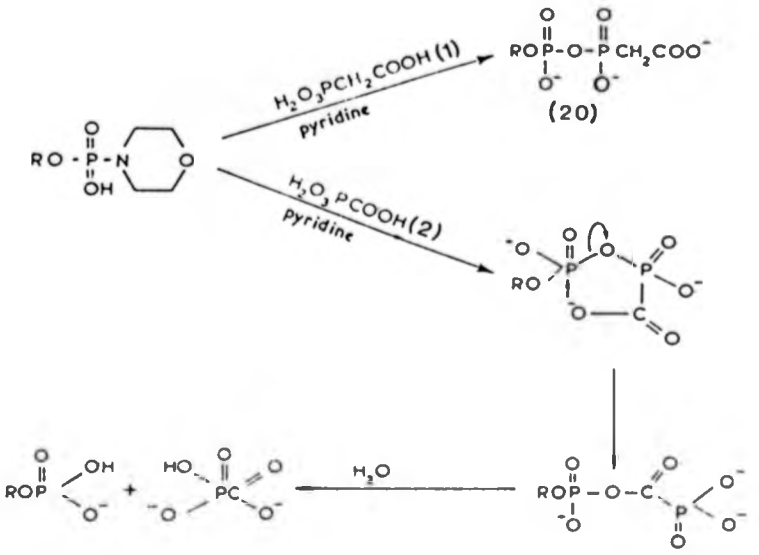


Fig. 4.3.i Reactions of adenosine 5'-phosphoromorpholidate with PAA and PFA.
R = adenosine

slow. Marked differences between the rates of intramolecular hydrolysis of phosphonate esters have been observed depending on the size of ring involved in the cyclic reaction. Thus, diethyl 2-carboxymethylphenylphosphonate undergoes intramolecular hydrolysis 10^5 times more slowly than diethyl 2-carboxyphenylphosphonate at pH 3.0 and 79.5°C (Fig. 4.3.ii) (Blackburn and Brown, 1969). The C-ethyl ester of PFA cannot decompose *via* a cyclic reaction as the carboxyl group is blocked. Hence, the formation of the pyrophosphate bond in the nucleoside triphosphate analogue can be observed.

(a) (b)

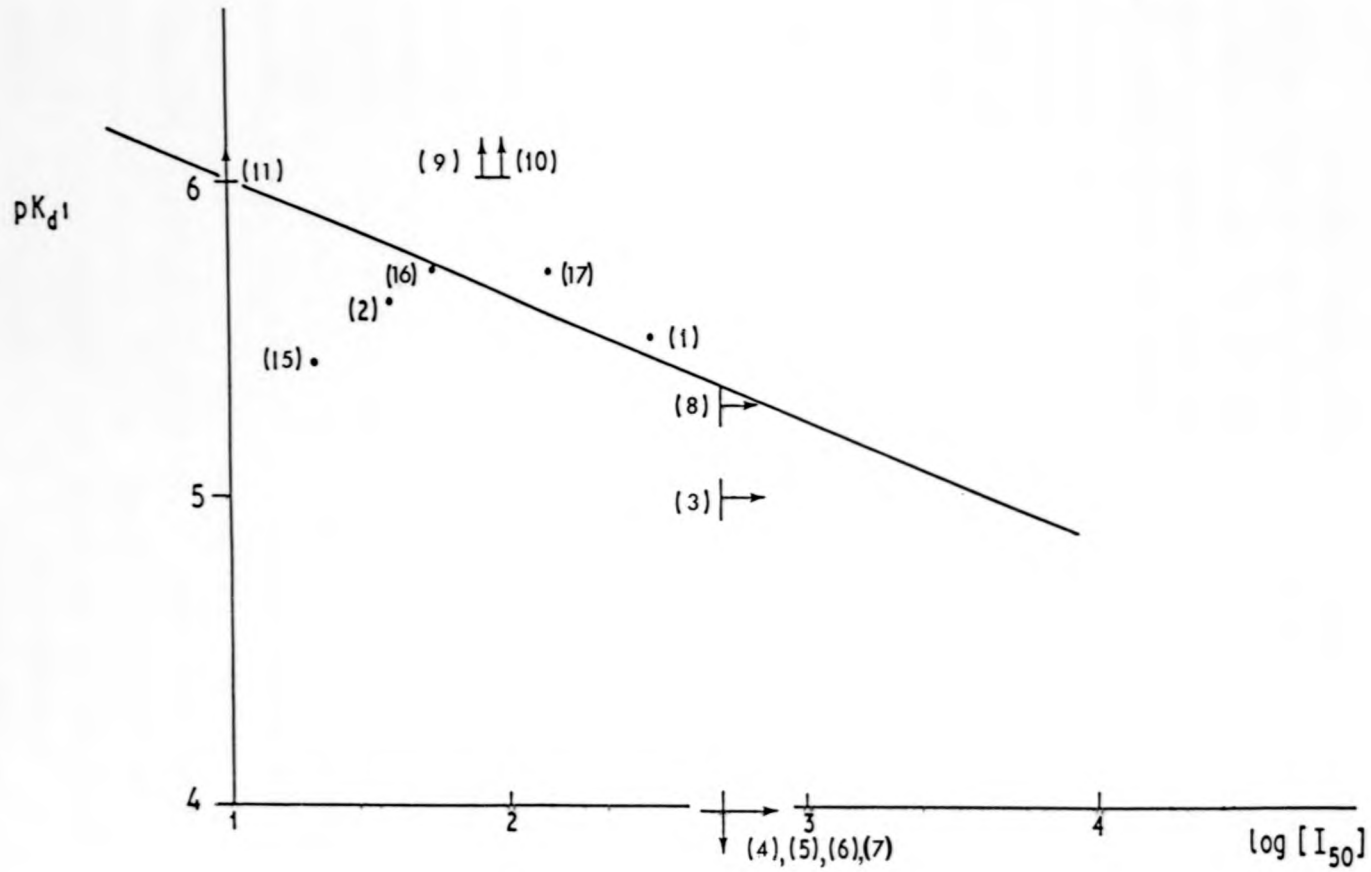
Fig. 4.3.ii (a) Diethyl 2-carboxymethylphenylphosphonate
(b) Diethyl 2-carboxyphenylphosphonate

Little data on the metal chelating properties of pyrophosphate analogues have been published. We have therefore, determined by gel filtration a dissociation constant pK_d, (Table 4.2.1) for complexes formed between zinc ions and pyrophosphate analogues at pH 8.0, the pH at which the RNA polymerase assays are carried out. We do not attempt to specify which ligand species are involved in complex formation as more than one species may be present and pK_d, is merely an indication of the strength of the interaction between the analogues and zinc ions at this pH. However, our pK_d, values do not differ markedly from the pK_d values reported for the fully ionised species of PAA [5.3] (Stünzi and Perrin, 1979) and dichloromethylenebisphosphonic acid [6.7] (Dietsch *et al.*, 1976). Thus, the gel filtration method was found to be an efficient and rapid method for the determination of stability constants. Several other methods including ^{31}P n.m.r., potentiometry, ion exchange and spectrophotometry were also evaluated for the determination of pyrophosphate analogue - zinc ion

stability constants. However, difficulties with ease and rapidity of operation and reproducibility of results effectively precluded their use in this particular study. We find that there is a correlation between the $pK_{d'}$ of an analogue and its effectiveness as an inhibitor of the RNA polymerase activity of influenza (Fig. 4.3.iii). Thus, PAA and PFA have relatively high $pK_{d's}$ and are inhibitors of the polymerase whereas (3) to (7) have lower $pK_{d's}$ and are ineffective inhibitors. Steric factors appear to be involved as PFA which can form a metal chelate with a five-membered ring is a more effective inhibitor than PAA which forms a chelate with a six-membered ring which in turn is a more effective inhibitor than 3-phosphonopropionic acid (4) which would form a chelate with a seven-membered ring. The correlation between chelate ring size and complex stability is as predicted from experience with other ligands.

A correlation between $pK_{d'}$ and inhibitor effectiveness also seems to exist for methylenebisphosphonates. Thus methylenebisphosphonate (8) which has a lower $pK_{d'}$ than the halogenated analogues (9) to (12) is an ineffective inhibitor whereas (9) to (12) are effective inhibitors. Interestingly, carbonylbisphosphonate (15) is a more effective inhibitor of the polymerase than (8) though it has a similar $pK_{d'}$. Here, steric factors may be important as the P-C-P bond angle in these two compounds should be very different. Inorganic pyrophosphate (17) and imidobisphosphate (16) have high $pK_{d's}$ and are effective inhibitors.

Fig. 4.3.iii Relationship between pK_d, (the ability of pyrophosphate analogues to bind zinc ions at pH 8.0) and their effectiveness as inhibitors of influenza (A/X-49) RNA polymerase.



I_{50} = Concentration [μM] producing 50% inhibition.

Although ethane-1-hydroxy-1,1-bisphosphonate (13) forms strong complexes with zinc ions in solution it is not an effective inhibitor of influenza RNA polymerase. This seemingly anomalous result can possibly be explained by the presence of a third chelation site. Analysis of x-ray crystallographic data (Barnett and Strickland, 1979) by molecular graphics indicates that the hydroxyl group of the hydroxyethyl moiety would be unable to bind to the zinc ion involved in the six-membered chelate ring but it could be involved in chelation to a second zinc ion in solution which would result in a stronger overall complex formation. This same rationale can also be applied to (18).

The inhibition of some zinc metalloenzymes (e.g. bovine liver glutamate dehydrogenase and bovine carboxypeptidase) by chelating agents can be reversed by the addition of excess zinc ions (Vallee, 1960). However, reversal of inhibition is highly dependent upon the relative stability constants of the inhibitor for polypeptide bound zinc and free zinc. Furthermore, systems containing metal ions and complexing agents in excess constitute metal ion buffer systems in which the free metal ion concentration is controlled by the other components. In particular, proteins contain several metal ion complexation sites ($-NH_2$, $-COO^-$, $-S^-$) which can effectively decrease the concentration of free metal ions and this may help to explain why it is frequently not possible to reverse the inhibitory effects of chelating agents by the addition of free

metal ions (Vallee, 1960).

The inhibition of influenza RNA polymerase by PFA could not be reversed by the addition of zinc ions to the reaction mixes and indeed in control reactions zinc ions themselves were found to be inhibitory. In a further attempt to reverse inhibition, PFA was added as its zinc salt (formed by ion exchange chromatography). However, this was found to be an even more effective inhibitor than the free drug. It is possible that the inhibitory effect of ZnPFA is two fold: on dissolution in the reaction mixture the salt dissociates yielding free zinc ions which bind to metal complexation site(s) on the enzyme and free PFA which binds to the pyrophosphate binding site.

Billards and Peets (1974) demonstrated that an excess of dithiothreitol (DTT) was capable of reversing the inhibitory effects of selenocystine and postulated that the influenza RNA polymerase was very sensitive to compounds which react with -SH groups. In control reactions, DTT at a concentration of 5 mM stimulated the incorporation of [^{3}H]-UMP into acid-precipitable product by 50%. However, no significant change in the inhibition profile of PFA was observed when DTT was omitted from the reaction mixes suggesting that -SH bonds do not play a major role in PFA inhibition.

The complexity of the assay system makes interpretation of the enzyme kinetics of the polymerase reaction difficult (for example, Lineweaver and Burk plots of the inhibition of this enzyme activity were curved). Studies

with active preparations of influenza RNA polymerase free from RNA and other proteins might help to establish the exact mode of action of pyrophosphate analogues. Upon the evidence amassed to date we suggest that pyrophosphate analogues inhibit influenza RNA polymerase by complexing with an essential zinc ion at the active site of the enzyme thus preventing the binding of nucleoside triphosphates or the release of inorganic pyrophosphate once the internucleotide bond has been formed by the enzyme. A possible reason for the differential sensitivities of various polymerases (e.g. DNA polymerase α) to these analogues may be that the environment around the essential zinc ion is such that an effective inhibitory complex cannot be formed in certain enzymes.

APPENDIX I

AMMONIA CHEMICAL IONISATION MASS SPECTRA OF ESTERS AND AMIDES OF OXYACIDS OF PHOSPHORUS

AI.1 INTRODUCTION

The electron impact (EI) mass spectra of esters of oxyacids of phosphorus have been widely studied and detailed fragmentation patterns for many esters have been published (Gillis and Occolowitz, 1972; Meyerson *et al.*, 1980). However, under standard EI conditions the interaction in the ion source between energetic electrons and sample molecules having ionisation potentials of 7-10 eV produces molecular ions that contain a considerable amount of excess energy which is largely dispersed through fragmentation. Consequently, the ion current carried by the molecular ion and other high molecular weight ions in the EI spectra of many phosphorus esters and amides is often much less than 1% of the total ion current, making identification and analysis of unknown compounds difficult. Chemical ionisation (CI) mass spectrometry has been suggested as a means of overcoming this problem (Sass and Fisher, 1979).

In CI mass spectrometry, a reagent gas such as methane is introduced into the ion source so as to achieve a reagent gas to sample ratio of 10^3 to 10^4 to 1. Under such conditions the principal process carried out by the energetic electrons in the ion source is ionisation of

the reagent gas. For methane, ionisation is followed by ion-molecule interactions which yield two dominant ionic species, CH_5^+ and $C_2H_5^+$. These ions can then ionise sample molecules (M) present in the source by means of proton transfer [1].

$$M + CH_5^+ \rightarrow MH^+ + CH_4 \qquad [1]$$

Ionisation by proton transfer is a considerably less energetic process than ionisation by electron-impact. Consequently, the degree of fragmentation which occurs under CI conditions is generally much less than that which occurs under EI conditions.

The degree of fragmentation which occurs can be controlled further by the choice of reagent gas. For example, isobutane as reagent gas will give rise to MH^+ ions which undergo far less fragmentation than is observed when methane is used as reagent gas. This is because the dominant ionic species produced from isobutane is the t-butyl ion which is a far weaker proton donor than the CH_5^+ ion. The use of ammonia as reagent gas results in still milder ionisation conditions and hence less fragmentation. In addition to the MH^+ ion the adduct ion $[M + NH_4]^+$ is also occasionally observed, its appearance being dependent upon the conditions employed and the basicity of the sample (Keough and DeStefano, 1981).

The CI mass spectra have been described for some phosphonates using hydrocarbons as reagent gases (Sass and Fisher, 1979) and this study extends these

initial experiments to esters and amides of other oxyacids of phosphorus using ammonia as reagent gas.

AI.2 EXPERIMENTAL

Unless otherwise stated, all compounds were commercially available. Tetra-isopropyl esters of halogenated methylenebisphosphonates were prepared by the method of Quimby *et al.* (1967). Diethyl phosphonoacetic acid was prepared by the method of Clayton *et al.* (1979).

Mass spectra were obtained using an MS80 mass spectrometer with a DS55 data system (Kratos Analytical Instruments). CI mass spectra were recorded using ammonia as reagent gas at a pressure of 2×10^{-4} Torr in the source housing (equivalent to approximately 0.1 Torr in the source). Electron energy was 55 eV. The ion source temperature was 200°C and the emission current 500 μA. EI mass spectra were obtained at 70 eV and a source temperature of 200°C. Samples were introduced *via* a solids probe in the usual manner.

AI.3 RESULTS AND DISCUSSION

AI.3.1 Phosphonates

The CI spectra obtained for compounds 1-10 (Table AI.3.i) were similar to those obtained previously for phosphonates with hydrocarbons (Sass and Fisher, 1979), the major high molecular weight signal being due to the protonated molecular ions $[M + 1]^+$. Useful structural

Table A1.3.1 Compounds Studied

	Name	Formula	Elemental Composition	Mol. Wt.
	PHOSPHONATES			
1	Tetra-isopropyl methylene bisphosphonate	$(Me_2CHO)_2P(O)CH_2P(O)(OCHMe_2)_2$	$C_{13}H_{30}O_6P_2$	344
2	Tetra-isopropyl monochloromethylene bisphosphonate	$(Me_2CHO)P(O)CHClP(O)(COCHMe_2)_2$	$C_{13}H_{29}ClO_6P$	378 380
3	Tetra-isopropyl monobromomethylene bisphosphonate	$(Me_2CHO)P(O)CHBrP(O)(OCHMe_2)_2$	$C_{13}H_{29}BrO_6P$	422 424
4	Tetra-isopropyl dibromomethylene bisphosphonate	$(Me_2CHO)P(O)CBr_2P(O)(OCHMe_2)_2$	$C_{13}H_{28}Br_2O_6P_2$	500 502,504
5	Triethyl phosphonoacetate	$(EtO)_2P(O)CH_2COOEt$	$C_8H_{17}O_5P$	224
6	Triethyl 2-phosphonopropionate	$(EtO)_2P(O)CHMeCOOEt$	$C_9H_{19}O_5P$	238
7	Diethyl phosphonoacetonitrile	$(EtO)_2P(O)CH_2CN$	$C_6H_{12}NO_3P$	177
8	Diethyl allylphosphonate	$(EtO)_2P(O)CH_2CH=CH_2$	$C_7H_{15}O_3P$	178
9	Benzyl diethyl phosphonoformate	$(EtO)_2P(O)COOCH_2Ph$	$C_{12}H_{17}O_5P$	272
10	Diethyl phosphonoacetic acid	$(EtO)_2P(O)CH_2COOH$	$C_6H_{13}O_5P$	196
	PHOSPHATES			
11	Trimethylphosphate	$(MeO)_3PO$	$C_3H_9O_4P$	140
12	Triethyl phosphate	$(EtO)_3PO$	$C_6H_{15}O_4P$	182
13	Tri-isopropyl phosphate	$(Me_2CHO)_3PO$	$C_9H_{21}O_4P$	224
14	Tri-n-butyl phosphate	$(BuO_3)PO$	$C_{12}H_{27}O_4P$	266
15	Triphenyl phosphate	$(PhO)_3PO$	$C_{18}H_{15}O_4P$	326
	PHOSPHITES			
16	Trimethyl phosphite	$(MeO)_3P$	$C_3H_9O_3P$	124
17	Triethyl phosphite	$(EtO)_3P$	$C_6H_{15}O_3P$	166
18	Triphenyl phosphite	$(PhO)_3P$	$C_{18}H_{15}O_3P$	310
	PHOSPHORAMIDATES			
19	Di-isopropyl phosphoramidate	$(Me_2CHO)_2P(O)NH_2$	$C_6H_{16}NO_3P$	181
20	Hexamethyl phosphoramide	$(Me_2N)_3PO$	$C_6H_{18}N_3OP$	179

data could be obtained from ammonia CI spectra. For example, the spectrum of tetra-isopropyl methylenebis-phosphonate (1) (Fig.AI.3.1) contained peaks at m/z 345 (6.6%), 303 (4.0%), 261 (6.2%), 219 (3.2%) and 117 (3.5%), indicating the successive loss of C_3H_6 (42 u) and confirming the presence of a tetra-isopropyl ester (Table AI.3.ii). The corresponding signals were scarcely discernible in the EI spectrum of 1. The CI spectra of the tetra-isopropyl esters of monochloro-(2), monobromo-(3) and dibromo-(4)-methylenebisphosphonates all showed similar families of peaks consisting of the protonated molecular ion and ions derived from the successive loss of propene residues. As might be expected, owing to the presence of halogen atoms, the protonated molecular ion and related ions in 2 were doublets (ratio 1:2:8), doublets (ratio 1:1) for 3 and triplets (ratio 1:2:1) for 4 (Table AI.3.ii).

Structural information could also be obtained from the CI mass spectra of the ethyl esters 5-10. In addition to the protonated molecular ion, other major ions corresponding to the successive loss of 28 u (presumably ethene) were present, confirming the presence of P-O-C_2H_5 residues. The CI mass spectra of 5 and 6 also showed ions corresponding to the loss of ethanol from the protonated molecular ion. This loss must involve the $COOC_2H_5$ group as this fragmentation does not occur in compounds 7-10. The CI mass spectrum of the carboxylic acid diethyl phosphonoacetic acid (10) showed a peak due to $[M + NH_4]^+$ m/z 214 (4.2%) as well

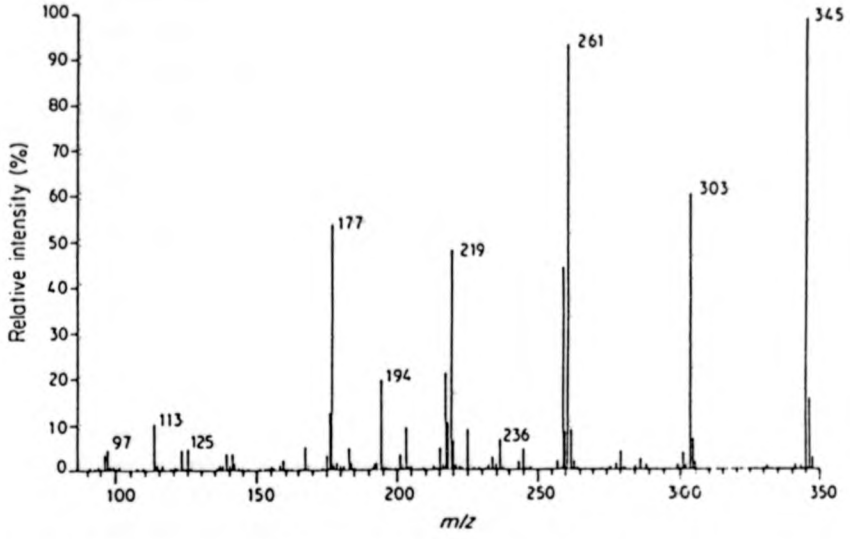


Fig AI.3.1. Ammonia CI mass spectrum of tetra-isopropyl methylene bisphosphonate (1).

Table AI.3.ii Principal Ions and % Total Ion Current Carried in Ammonia CI Mass Spectra of Esters and Amides of Oxyacids of Phosphorus

	PHOSPHONATES
1	345 (6.6%), 303 (4.0), 261 (6.2), 219 (3.2), 177 (3.5)
2	381 (2.3%), 379 (6.6), 345 (3.2), 339 (1.9), 337 (5.3), 297 (1.2), 295 (3.6), 255 (0.6), 253 (1.7), 213 (0.3), 211 (1.0)
3	425 (1.3%), 423 (1.3), 383 (1.3), 381 (1.3), 345 (2.9), 341 (0.8), 339 (0.8), 299 (0.3), 297 (0.3), 257 (0.3), 255 (0.3)
4	505 (1.7%), 503 (3.4), 501 (1.8), 463 (2.6), 461 (5.2), 459 (2.8), 421 (1.8), 419 (3.5), 417 (1.9), 379 (0.6), 377 (1.2), 375 (0.8)
5	225 (42.0%), 197 (9.5), 179 (5.3), 151 (3.9)
6	239 (29.6%), 211 (3.6), 193 (8.0), 183 (1.2)
7	195 (16.8%), 178 (52.0), 150 (34), 122 (1.1)
8	180 (11.3%), 179 (17.0), 178 (6.2), 167 (8.6), 151 (4.4), 134 (3.3), 123 (2.1)
9	273 (13.3%), 245 (0.3), 229 (12.6), 217 (0.2), 181 (8.8), 91 (29.3)
10	214 (4.2%), 197 (29.5), 183 (5.8), 169 (1.9), 153 (11.7)
	PHOSPHATES
11	141 (23.3%), 110 (6.1), 94 (1.6), 79 (0.6)
12	183 (38.9%), 155 (20.0), 127 (5.4), 99 (0.6)
13	225 (22.8%), 183 (14.4), 141 (8.2), 99 (1.1)
14	267 (34.4%), 211 (14.8), 155 (8.0), 99 (1.6)
15	327 (40.3%), 233 (1.8), 170 (2.0)
	PHOSPHITES
16	125 (10.8%), 111 (11.4), 94 (1.9)
17	167 (47.2%), 139 (20.9), 121 (2.5), 111 (2.2)
18	311 (10.4%), 235 (8.0), 217 (10.1), 153 (2.9), 94 (8.5)
	PHOSPHORAMIDATES
19	182 (33.5%), 140 (14.6), 124 (3.9), 98 (14.9)
20	180 (22.3%), 136 (5.9), 135 (10.0), 92 (1.8)

Table AI.3.ii Principal Ions and % Total Ion Current Carried in Ammonia CI Mass Spectra of Esters and Amides of Oxyacids of Phosphorus

	PHOSPHONATES
1	345 (6.6%), 303 (4.0), 261 (6.2), 219 (3.2), 177 (3.5)
2	381 (2.3%), 379 (6.6), 345 (3.2), 339 (1.9), 337 (5.3), 297 (1.2), 295 (3.6), 255 (0.6), 253 (1.7), 213 (0.3), 211 (1.0)
3	425 (1.3%), 423 (1.3), 383 (1.3), 381 (1.3), 345 (2.9), 341 (0.8), 339 (0.8), 299 (0.3), 297 (0.3), 257 (0.3), 255 (0.3)
4	505 (1.7%), 503 (3.4), 501 (1.8), 463 (2.6), 461 (5.2), 459 (2.8), 421 (1.8), 419 (3.5), 417 (1.9), 379 (0.6), 377 (1.2), 375 (0.8)
5	225 (42.0%), 197 (9.5), 179 (5.3), 151 (3.9)
6	239 (29.6%), 211 (3.6), 193 (8.0), 183 (1.2)
7	195 (16.8%), 178 (52.0), 150 (34), 122 (1.1)
8	180 (11.3%), 179 (17.0), 178 (6.2), 167 (8.6), 151 (4.4), 134 (3.3), 123 (2.1)
9	273 (13.3%), 245 (0.3), 229 (12.6), 217 (0.2), 181 (8.8), 91 (29.3)
10	214 (4.2%), 197 (29.5), 183 (5.8), 169 (1.9), 153 (11.7)
	PHOSPHATES
11	141 (23.3%), 110 (6.1), 94 (1.6), 79 (0.6)
12	183 (38.9%), 155 (20.0), 127 (5.4), 99 (0.6)
13	225 (22.8%), 183 (14.4), 141 (8.2), 99 (1.1)
14	267 (34.4%), 211 (14.8), 155 (8.0), 99 (1.6)
15	327 (40.3%), 233 (1.8), 170 (2.0)
	PHOSPHITES
16	125 (10.8%), 111 (11.4), 94 (1.9)
17	167 (47.2%), 139 (20.9), 121 (2.5), 111 (2.2)
18	311 (10.4%), 235 (8.0), 217 (10.1), 153 (2.9), 94 (8.5)
	PHOSPHORAMIDATES
19	182 (33.5%), 140 (14.6), 124 (3.9), 98 (14.9)
20	180 (22.3%), 136 (5.9), 135 (10.0), 92 (1.8)

as a large peak due to the protonated molecular ion (29.5%). Peaks due to loss of carbon dioxide (m/z 153, 11.7%) and ethene (m/z 169, 1.9%) from the protonated molecular ion were also present.

AI.3.2 Phosphates

Previous CI studies (Sass and Fisher, 1979) did not include phosphate esters although their EI mass spectra have been studied extensively (Occolowitz and Swan, 1966). For phosphate esters, as might be expected, the major high molecular weight ion in their CI mass spectra was $[M + 1]^+$. Again families of peaks could be observed in the CI spectra from which information could be deduced as to the nature and number of groups bonded to phosphorus. Thus, the trialkyl esters 12-14 gave $[M + 1]^+$ and ions due to the successive loss of three molecules of olefin (ethene, propene and butene respectively) (Fig. AI.3.2). Trimethyl (11) and triphenyl (15) phosphates showed little fragmentationin their CI mass spectra. The fragmentation of the trialkyl phosphates could be observed in their EI mass spectra. However, as the ion current carried by the molecular ion was less than 1% of the total ion current in all cases, the fragmentation patterns were much more difficult to observe than in the CI mass spectra.

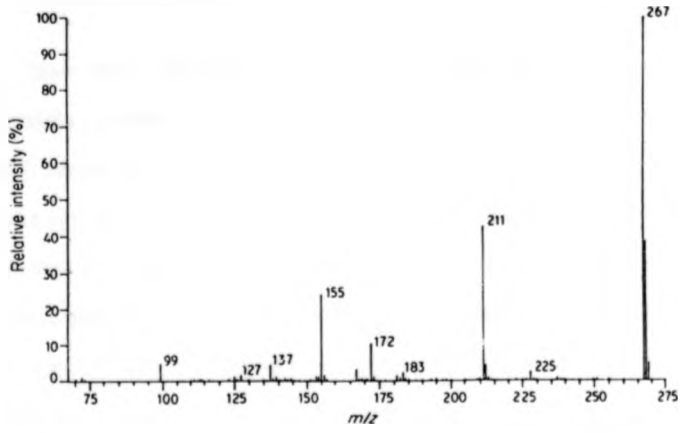


Fig AI.3.2. Ammonia CI mass spectrum of tri-*n*-butyl phosphate (13).

AI.3.3 Phosphites

Ammonia CI mass spectrometry was also useful for the detection of esters of oxyacids of trivalent phosphorus. In the three compounds tested (16-18), the protonated molecular ion carried at least 10% of the total ion current. For triethyl phosphite (17) peaks corresponding to the loss of two and not three molecules of ethene could be observed. There was little fragmentation observable in the CI mass spectrum of trimethyl phosphite (16), while the major fragmentation in the CI mass spectrum of triphenylphosphite (18) corresponded to the loss of phenol (m/z 94) from the protonated molecular ion.

AI.3.4 Phosphoramidates

The two phosphoramidates examined (19 and 20) showed strong peaks in their CI mass spectra due to protonated molecular ions. The major features in the CI mass spectrum of di-isopropyl phosphoramidate (19) were m/z 140 (14.6%) and 98 (14.9%) corresponding to the successive loss of two molecules of propene. In this case, the protonated molecular ion carried 33.5% of the ion current carried by the sample, whereas in the EI mass spectrum the protonated molecular ion (the major, high molecular weight ion) carried only 0.3% of the total ion current. The CI mass spectrum of hexamethyl phosphoramide (20) showed only one major peak, in addition to $[M + H]^+$ (22.3%), corresponding to the loss of 45 u, presumably Me_2NH.

AI.3.5 Methane Chemical Ionisation Mass Spectrometry

For comparison, a representative selection of compounds were also studied by methane CI mass spectrometry (Table AI.3.5).

The results show that, as predicted, far less fragmentation occurs under methane CI conditions than is observed under EI conditions. However, the percentage ion current carried by the $[M + H]^+$ ions is on the whole much lower than in the comparable ammonia CI mass spectra. Hence, ammonia CI would appear to be the method of choice for the identification and analysis of esters and amides of oxyacids of phosphorus.

Table AI.3.5 Principal Ions and % Total Ion Current Carried in Methane CI Mass Spectra of Esters and Amides of Oxyacids of Phosphorus

	PHOSPHONATES
1	345 (6.72%), 303 (1.89), 261 (2.59), 219 (2.64), 177 (2.94)
5	225 (5.11%), 197 (2.02), 179 (2.23), 151 (0.74)
6	239 (27.23%), 2.11 (1.09), 193 (6.50), 183 (0.44)
	PHOSPHATES
12	183 (4.93%), 155 (3.63), 127 (1.75), 99 (1.05)
14	267 (14.50%), 211 (7.21), 155 (7.51), 99 (15.11)
	PHOSPHITES
17	167 (10.90%), 139 (5.61), 121 (0.83), 111 (0.51)
	PHOSPHORAMIDATES
20	180 (17.30%), 136 (3.29), 135 (14.47), 92 (0.96)

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Determining the Stability Constant of a Metal Complex by Gel Chromatography

Let us consider the formation of a 1:1 metal complex, ML, between a metal ion, M, and a ligand, L, according to eqn. (1)

$$M + L \rightleftharpoons ML \tag{1}$$

For the sake of simplicity the charge on each species is not presented. The concentration stability constant, K, of the metal complex to be determined is given by

$$K = \frac{[ML]}{[M][L]} \tag{2}$$

in which [ ] represents the molar concentrations of M, L, and ML.

Various physiochemical methods, such as spectrophotometry, potentiometry, ion exchange methods, solvent extraction methods, solubility methods, etc., have usually been employed to determine the concentration or the activity of M, L, or ML (*1, 2*). If one of these activities or concentrations is experimentally determined, the other two can be calculated, which enables us to evaluate the stability constant of the metal complex. For example, if [ML] is determined spectrophotometrically, [M] and [L] will then be calculated by

$$[M] = [M]_t - [ML] \tag{3}$$

$$[L] = [L]_t - [ML] \tag{4}$$

in which $[M]_t$ and $[L]_t$, the respective total concentrations of metal and ligand, are known.

In this paper we describe the principle of a new technique that is based on a gel chromatographic method (*3–7*) and has been increasingly applied to the characterization of metal-ligand binding in the fields of inorganic (*8*), bioinorganic (*9–17*), and environmental (*18*) chemistry. This chromatographic method has an advantageous and unique characteristic over the conventional static (batchwise) equilibrium methods, in that [M] can be kept at a desired and predetermined value, $[M]_0$, throughout the experiment. In other words, the equilibrium solution being examined is "buffered" with respect to metal ion, as well as with respect to hydrogen ion, and [ML] and [L] are then dynamically adjusted to be in equilibrium with $[M]_0$ through the process of their chromatographic migration. Therefore, [M] is not required to be analyzed experimentally and one can evaluate the stability constant of the metal complex according to eqn., (5) by the measurement of [ML] that is in equilibrium with $[M]_0$.

$$K = \frac{[ML]}{[M]_0([L]_t - [ML])} \tag{5}$$

in which $[M]_0$ and $[L]_t$ are known. The only undetermined value, [ML], can be calculated by

$$[ML] = [M]_t - [M]_0 = a(A_{ob} - A_{M_0}) = a A_{ob} - b \tag{6}$$

where A_{ob} is the observed absorbance, that is the sum of the respective absorbances, A_{M_0} and A_{ML}, due to free metal ion and metal complex. a and b are constants. Equations (5) and (6) indicate that [ML] or K can be easily determined by measuring A_{ob} without any complicated analysis of the observed results. The atomic absorption method has been extensively and successfully employed to obtain reproducible results (*8, 10, 18*). Spectrophotometric methods are powerful if the absorption due to free ligand is negligible. The terms A_{ob} and A_{M_0} in eqn. (6) can also be translated into any kind of physicochemical responses of ML and M, such as are based on radioactivity measurement (*18*).

The most important point in practice of this approach is how to keep the concentration of free metal ion at a constant level during the equilibrium experiment. It seems very difficult or almost impossible to maintain [M] constant by the conventional static methods in which [M] is usually allowed to be dependent on $[M]_t$ and $[L]_t$ as indicated in eqns. (3) and (4). As will be mentioned below, however, a simple way to answer this requirement is by use of a gel chromatographic method which was first applied by Hummel and Dreyer (*3*) to the binding of 2′-cytidilic acid with ribonuclease and has subsequently been employed for estimation of the stability constants of various metal complexes (*6, 9*), with some progressive modification in practical procedure (*8, 11*). This technique enables us to observe directly the formation of ML complex that is in equilibrium with metal ion of the specified concentration, $[M]_0$.

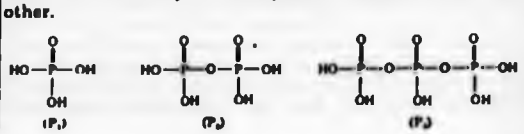
Gel Chromatography of Metal Ions and Ligands

Before we deal with the detailed discussion on the gel chromatographic determination of the stability constant of metal complex let us review briefly the gel chromatographic behavior of two constituents, a metal ion and a ligand (*6, 19*).

Gel chromatography is a form of liquid chromatography capable of separating solute molecules according to their size, and its basic principle has been explained in terms of sieving effect or steric exclusion (*4–7*). The separation of the components in a sample solution is usually carried out on a column packed with a gel or other porous material. It is a general elution pattern, though not always if there are side effects such as adsorption, that the solute molecules are eluted in the order of decreasing molecular size.

Although it was first applied mostly to the separation of very small molecules from very large ones, e.g., desalting from proteins, it has progressively been demonstrated to be widely applicable to the separation of not only a mixture of macromolecules but also a mixture of lower-molecular-weight species as small as hydrated metal ions. For example, the elution volumes of alkaline earth metal ions on a Sephadex G-15 column (Pharmacia Fine Chemicals AB) increased in the order, Mg < Ca ≈ Sr < Ba, which could be qualitatively correlated with the reverse sequence of the radii of these hydrated metal ions, i.e., Mg (4.28 Å) > Ca (4.12 Å) = Sr (4.12 Å) > Ba (4.04 Å) (*20*).

A more pronounced effect of steric exclusion has been shown for the elution behavior of inorganic polyphosphate anions with different degrees of polymerization (*6, 21*). For example, the elution volumes of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3), tetraphosphate (P_4), and more high polymers (P_n) decreased in the order $P_1 > P_2 > P_3 > P_4 > P_n$. A mixture of P_1, P_2, and P_3, shown below for their acids, can be easily and completely separated from each other.



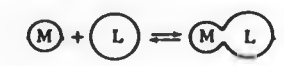
For the quantitative expression, the elution volume of a solute molecule, V_e, is represented in terms of some column parameters (*6*). For very large molecules that are completely excluded from the gel pores, V_e is equal to the interstitial volume of the column, V_0. For very small molecules that can penetrate into all parts of the gel pores, V_e is equal to the total liquid volume of the column, $V_i + V_0$. For molecules of intermediate size, the elution volume is given by

$$V_e = V_0 + K_d V_i \tag{7}$$

in which K_d is a distribution coefficient that depends on the molecular size and lies in the range, $0 \leq K_d \leq 1$.

Gel Chromatography of Metal Complex

In eqn. (8) a complexation reaction is shown schematically to illustrate the difference in size among a metal ion, a ligand, and a metal complex (*6, 21*)



(8)

in which the circles represent the sizes of the free metal ion, the free ligand, and the metal-ligand complex. It is assumed that if the size of the free metal ion is relatively very small compared with that of the free ligand, the size of the metal complex may be primarily determined by the contribution from the size of the ligand. In such a situation, the metal complex is expected to appear at or near the elution position of the free ligand. This speculation seems reasonable to a first approximation for polyatomic ligands such as inorganic polyphosphates, EDTA, nucleotides, proteins, etc. The elution volumes of magnesium ion and magnesium polyphosphate complexes, for example, decrease in the order, $Mg > MgP_2 > MgP_3 > MgP_4 > MgP_n$, which is in accord with the prediction from the increasing order in the ionic sizes, $P_2 < P_3 < P_4 < P_n$, or the decreasing order in the elution volumes, $P_2 > P_3 > P_4 > P_n$, of the corresponding polyphosphate anions (*8*). The metal complex in some cases tends to be eluted later than the free ligand (*6, 22, 23*), for which no satisfactory explanation has been given. It is conceivable that the size reduction of the metal complex, in contrast to the expectation from the molecular weight basis, may be caused by such effects as the chelation and the reduction in charge of the hydration layer.

It should be noted that if the ML complex is eluted with an eluent containing neither M or L, it will dissociate successively into its constituents during the passage through the column to give a complicated elution profile of M, L, and ML, depending on both the stability constant and kinetic factors (*19*). On the other hand, if an eluent contains a constant concentration of M and the complexation equilibrium is established rapidly so as not to permit the separation of ML and L, a well-defined peak of ML accompanied by L is expected to appear at the elution position that corresponds to the weighted average of the elution volumes of ML and L.

Determination of Stability Constant

For the sake of clarity in expression, the formation of a magnesium complex, MgL, will be hereafter presented in interpreting how one can determine the stability constant of a metal complex (*6, 8, 19*).

A gel chromatographic column packed with a tightly cross-linked gel, such as Sephadex G-10, G-15, and G-25 (Pharmacia Fine Chemicals AB) is pre-equilibrated with a buffered eluent containing a known concentration of magnesium ion, $[Mg]_0$. A known amount of a ligand, L, is dissolved in a solution of magnesium ion to prepare a sample solution. It is an important prerequisite that the total magnesium concentration in the sample be exactly the same as that in the eluent, if a Hummel and Dreyer pattern (Fig. 1) is to be obtained. This means that the concentration of free magnesium ion in the sample solution is reduced to a level lower than $[Mg]_0$ by an amount corresponding to the MgL complex formed.

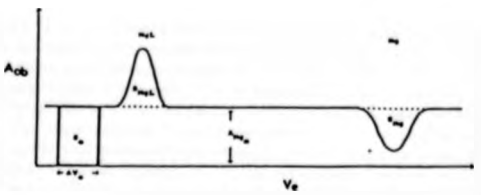


Figure 1. A schematic representation of Hummel and Dreyer pattern. According to eqn. (6) $[Mg]_0 = a\,A_{ob}$.

An aliquot of this sample solution is applied to the pre-equilibrated column and then eluted with the eluent used to preequilibrate the column. The MgL complex, along with L that is in a state of dynamic equilibrium with ML, migrates down the column more rapidly than the free magnesium ion so as to come out from the zone of magnesium deficiency. L and ML continue their travel through the pre-equilibrated column to be in contact with fresh free magnesium ions in the subsequent plates to form more MgL until a steady state is reached in equilibrium with $[Mg]_0$ that is the concentration of free magnesium ion in the mobile phase of the pre-equilibrated column. The resulting elution profile is schematically shown in Figure 1. The absorbance, A_{ob}, corresponding to the total magnesium concentration, $[Mg]_t = [Mg] + [MgL]$, is assumed to be monitored by atomic absorption methods and plotted against the elution volume (see eqn. (7)).

When MgL along with L emerges faster, the total concentration of magnesium in the effluent rises above the base line level to form a positive peak of MgL. Behind the MgL peak, A_{ob} continues to be constant and then decreases, at the elution position of free magnesium ion, to below the base line level to form a negative peak that corresponds to the amount of magnesium consumed to form MgL. The appearance of a pair of positive and negative peaks in the elution profile therefore provides a criterion of binding of M and L. It is evident that the height of the horizontal base line corresponds to $[Mg]_0$. The concentration level of free magnesium in the zone of MgL complex has been interpreted to be equal to that in the horizontal region, provided that the sample concentration is reasonably low in comparison with the concentration of background electrolyte in the eluent (*8, 11*). In other words, the peak area of MgL above the base line, S_{MgL}, is a direct measure of the total amount of MgL complex that is in equilibrium with free magnesium whose concentration is $[Mg]_0$. It is also noted that S_{MgL} should be equal to the area of the negative peak, S_{Mg}. The peak area S_{MgL} or S_{Mg} can be easily translated into the amount of MgL, Q_{MgL}, on the basis of the standardized area, S_0, that is regarded as an internal standard to be calibrated against the known amount of magnesium, $\Delta V_e [Mg]_0$. Equation (5) thus can be rewritten as follows to simplify the calculation of the stability constant

$$K = \frac{Q_{MgL}}{[Mg]_0 (Q_{L_t} - Q_{MgL})} \tag{9}$$

where Q_{L_t} represents the total amount of L applied.

The ratio of Q_{MgL} to Q_{L_t} is designated as $\bar{n}$ and is correlated to K and $[Mg]_0$ by

$$\bar{n} = \frac{Q_{MgL}}{Q_{L_t}} = \frac{K\,[Mg]_0}{1 + K\,[Mg]_0} \tag{10}$$

The Hummel and Dreyer method in Figure 1 has a great advantage that Q_{MgL} can be estimated as well from S_{Mg} even when S_{MgL} can not be quantitatively determined by the spectrophotometric method owing to the interference by L that is also at the elution position of MgL. The exact measurement of S_{Mg}, however, is often difficult because the negative peak of free metal ions tends in general to broaden

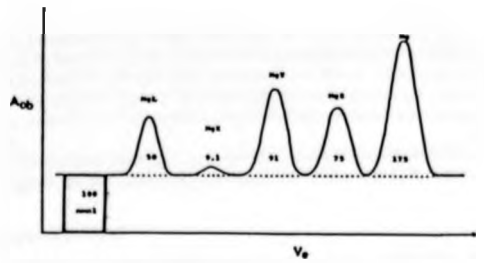


Figure 2. A schematic representation of an elution profile for a mixed solution of magnesium ion and four ligands, i.e., L, X, Y, and Z.

markedly if the metal ions are susceptible to adsorption. Another disadvantage of the S_{Mg} measurement is that S_{Mg} can not be directly translated into Q_{MgL} if more than one ligand is contained in a sample to form various metal complexes (Fig. 2). To minimize the analytical error resulting from the peak area measurement it is recommended that the relatively well-defined peak of MgL complex be measured by employment of specific methods such as atomic absorption (*8*) and, for metals such as Zn-65, radioactivity measurement (*18*). In such instances the preparation of a sample solution becomes more convenient because magnesium concentration in the sample solution need not be the same as that in the eluent. It is in reasonable excess so as to obtain a positive peak at the elution position of free magnesium ion (Fig. 2), in contrast to the negative peak in Figure 1.

A schematic profile to be expected is shown in Figure 2 when a mixed solution (1 ml) containing each 100 nmole of four ligands, i.e., L, X, Y, and Z that are different in size from each other, is assumed to be eluted with an eluent; $[Mg]_0 = 10^{-5}\ M$. The sample solution also contains 410 nmole of total magnesium that is 400 nmole in excess of that in 1 ml of the eluent. If the stability constants of four magnesium complexes are given as 1.0×10^5 for MgL, 1.0×10^4 for MgX, 1.0×10^6 for MgY, and 3.0×10^5 for MgZ, one can expect the amounts of the respective magnesium complexes, Q_{MgL}, Q_{MgX}, Q_{MgY}, and Q_{MgZ}, that are numerically (in nmole) shown in Figure 2 for four peaks of magnesium complexes, as well as for a positive peak of free magnesium ion. In other words, one can easily calculate the stability constant, for example, of MgZ complex according to eqns. (9) and (11), if Q_{MgZ} is given.

$$K = \frac{75}{1.0 \times 10^{-5}(100 - 75)} = 3.0 \times 10^5 \qquad (11)$$

There is an example of the exercise in calculation of this class which is based on the peak area measurement on the chart of a magnesium diphosphate complex (*8*) whose log K value obtained by other static methods (*24*) has been reported to be 5.42.

Numerical values in Figure 2 also indicate that the peaks for magnesium complexes increase at the expense of the peak of free magnesium ion. It should be noted, however, that the amount of each magnesium complex is dependent on $[Mg]_0$ but does not depend on both the amount of free magnesium ion in the sample or the peak area of free magnesium ion and the presence of other ligands. Therefore, Q_{MgL} values obtained by both methods in Figures 1 and 2 should be, in principle, equal to each other, so long as $[Mg]_0$ is the same.

According to eqn. (10) Q_{MgL} or $\bar{n}$ depends on $[Mg]_0$. If $[Mg]_0$ is lowered from $1.0 \times 10^{-5}\ M$ to $1.0 \times 10^{-6}\ M$, the values of Q_{MgL}, Q_{MgX}, Q_{MgY}, and Q_{MgZ} will accordingly decrease from 50, 9.1, 91, and 75 to 9.1, 1.0, 50, and 23, respectively, to result in the increase in the amount of free magnesium ion from 175 to 317.

The ligand concentration is usually required to be very low, in comparison to the concentration of background electrolyte, to avoid the complexity caused by the Donnan effect (*11, 13, 25*). In the accurate analysis of such a dilute sample solution it is important to know how to choose $[Mg]_0$ for sensitive detection and how to minimize the analytical error. It is very convenient in practical use to remember that, if $K[Mg]_0 = 1$, 50% of the total ligand can be detected as MgL and the relative error in estimating K can be predicted to be twice the relative error in the measurement of Q_{MgL}, which is acceptable. On the other hand, at $K[Mg]_0 = 10$, the advantage that about 90% of the total ligand can be detected as MgL can not overcome the drawback of the unfavorable relative error in K that amounts to approximately ten times that in Q_{MgL}.

Many investigations have been reported on the binding of more than one metal to a ligand (*12–18*), including the competitive or cooperative binding of more than one kind of metal (*11, 26*). In order to avoid the complexity in the description of the principle, however, the information from these interesting reports can not be presented in this paper.

The gel chromatographic method has application from the analytical viewpoint. It is evident from eqn. (10) that Q_{MgL} or S_{MgL} can be calibrated against Q_L to be determined. For example, gel chromatographic technique combined with an atomic absorption flow detector has been successfully applied to the automatic and sensitive analysis of a mixture of various polyphosphates (*8*). This method will be in principle applicable to the detection of any kinds of ligands which combine with a given metal ion that can be sensitively detected by atomic absorption or other selective methods.

Questions on the Mechanism

We have received many questions about this new technique from those who are familiar with the conventional static equilibrium methods in determining the stability constant. Most of these questions are based on a doubt as to whether or not the equilibrium we can observe in such a dynamic transport process can be regarded as the same as that in a static equilibrium system. One of the important questions is how one can realize that MgL and L are in equilibrium with $[Mg]_0$. As has been pointed out in the original paper of Hummel and Dreyer (*3*) the establishment of a steady state in equilibrium with $[Mg]_0$ is visually reflected on the appearance of a base line level corresponding to $[Mg]_0$ between the peaks of MgL and Mg.

The second question is whether or not this technique is also applicable, as shown in Figure 2, to metal complexes that are not large enough to be completely excluded from the gel phase. This doubt may arise, unfortunately, from the confusing description in the literature by some gel chromatographers who stressed the preferable use of the gel type that permits the complete exclusion of MgL and L. As has been noted by Determann and Brewer (*5*), it is not necessary for the ligand to be excluded completely from the gel phase. A prerequisite is that the degree of separation between the peaks of MgL and Mg be good so as to permit the appearance of a base line corresponding to $[Mg]_0$ between two peaks. This technique, for example, can be successfully applied to the relatively small ligands such as EDTA and diphosphate anions whose K_d values in eqn. (7) are not zero (*8, 23*).

The third question is about the effect of the stationary phase on the complexation equilibrium, which is related in a complicated manner to the above two questions. This question seems out of consideration for MgL and L, whose K_d values are zero, and which is present only in the mobile phase. To be considered is the case for smaller MgL and L that are distributed not only in the mobile phase but also, in part, in the stationary phase. In order to make the answer as simple as possible, let us remember that, according to a random go-and-stop concept in chromatography (*27*), a solute molecule migrates down the column only when it is in the mobile phase, while its down-stream motion in the stationary phase is halted. This means that the effluent we can analyze contains only the equilibrium components in the mobile phase at the last plate of the column. The stationary gel phase plays an important

role in separating MgL and Mg to make a steady state in equilibrium but does not affect the complexation equilibrium in the mobile phase that we can determine. The interstitial mobile phase (space) between gel particles may be regarded as a vessel in which a static equilibrium experiment is carried out.

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APPENDIX III

SOURCES OF MATERIALS

Adenosine 5'-phosphoromorpholidate	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Adenylyl-(3'-5')-guanosine	Boehringer, Mannheim, G.F.R.
Adenylyl-imidobiphosphate	Boehringer, Mannheim, G.F.R.
3-Amino-1-hydroxy-propane-1,1-bisphosphonate	Koch Light Laboratories, Colnbrook, Bucks., U.K.
Bovine pancreatic DNase I	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Calf Thymus DNA polymerase I	P-L Biochemicals, Milwaukee, Wisconsin, U.S.A.
Cellulose F_{254} TLC plates	E. Merck, Darmstadt, G.F.R.
Chromatography paper (3 MM)	Whatman Ltd., Maidstone, Kent, U.K.
DEAE cellulose	Whatman Ltd., Maidstone, Kent, U.K.
Dulbecco's Modification of Eagles Medium (DMEM x 10)	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
Dowex 50	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Ethane-1-hydroxy-1,1-bisphosphonate	Koch Light Laboratories Ltd., Colnbrook, Bucks., U.K.
GF/C discs (2.5 cm)	Whatman Ltd., Maidstone, Kent, U.K.
L-Glutamine	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
Glasgow Modification of Eagles Medium (GMEM x 10)	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
^3H_2O	Amersham International plc, Amersham, Bucks., U.K.
Imidobisphosphate	Boehringer, Mannheim, G.F.R.
Iodotrimethylsilane	Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.
Newborn calf serum (NCS)	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
Non-essential amino acids (NEAA)	Flow Laboratories, Irvine, Ayrshire, U.K.
Nonidet P-40	Sigma (London) Chemical Co., Poole, Dorset, U.K.
(deoxy)Nucleoside triphosphates	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Penicillin/Streptomycin	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.

3-Phosphonopropionic acid	Fluorochem Ltd., Glossop, Derbyshire, U.K.
Salmon sperm DNA	Calbiochem, La Jolla, California, U.S.A.
Sephadex G-10	Pharmacia Fine Chemicals, Uppsala, Sweden
Tetraethyl dimethylaminomethylene-bisphosphonate	Lancaster Synthesis Ltd., Morecambe, Lancashire, U.K.
Tetraisopropyl methylenebisphosphonate	Lancaster Synthesis Ltd., Morecambe, Lancashire, U.K.
Tetrasodium pyrophosphate	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.
Triethyl phosphonoacetate	Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.
Triethyl 2-phosphonopropionate	Roche Products Ltd., Welwyn Garden City, Herts., U.K.
[Methyl-^{3}H]Thymidine 5'-triphosphate (42 ci/mmol)	Amersham International plc, Amersham, Bucks., U.K.
[5,6-^{3}H]Uridine 5'-triphosphate	Amersham International plc, Amersham, Bucks., U.K.
Zinc chloride ('SpectroSol')	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

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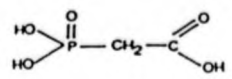
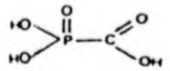
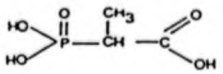
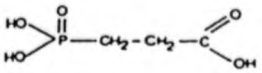
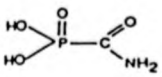
STRUCTURES OF COMPOUNDS STUDIED

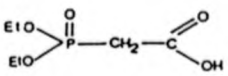
1. 

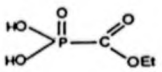
2. 

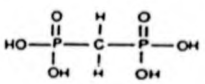
3. 

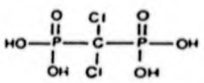
4. 

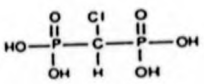
5. 

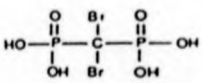
6. 

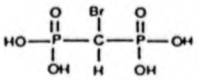
7. 

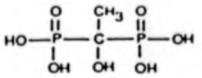
8. 

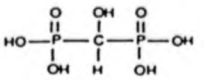
9. 

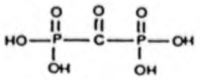
10. 

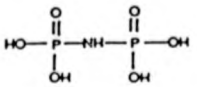
11. 

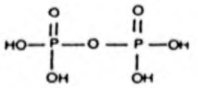
12. 

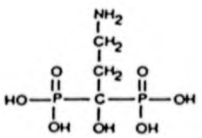
13. 

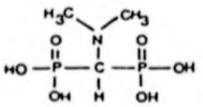
14. 

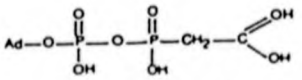
15. 

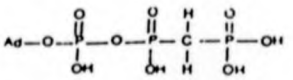
16. 

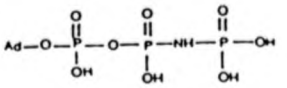
17. 

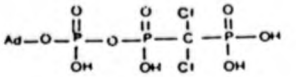
18. 

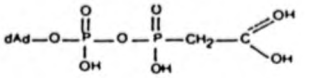
19. 

20. 

21. 

22. 

23. 

24. 

Ad = adenosine,
dAd = deoxyadenosine.

Ammonia Chemical Ionization Mass Spectra of Esters and Amides of Oxyacids of Phosphorus

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Chemical ionization mass spectrometry using ammonia as the reagent gas has been carried out with esters and amides of a variety of oxyacids of phosphorus (phosphates, phosphonates, phosphites and phosphoramidates). In all cases, the protonated molecular ion is a major species in the spectrum and the percentage of the total ion current carried by these protonated molecular ions is always considerably greater than that carried by the molecular ions in the corresponding electron impact mass spectra. In the chemical ionization mass spectra only limited fragmentation of the protonated molecular ion occurs from which useful information on the structure of phosphorus derivatives may be inferred.

INTRODUCTION

The electron impact (EI) mass spectra of esters of oxyacids of phosphorus have been widely studied and detailed fragmentation patterns for many esters have been published.[1,2] However, the ion current carried by the molecular ion and other high molecular weight ions in the EI spectra of many phosphorus esters and amides is often much less than 1% of the total ion current, making identification and analysis of unknown compounds difficult. Chemical ionization (CI) mass spectrometry has been suggested as a method to overcome this problem and CI spectra have been described for some phosphonates using hydrocarbons as reagent gases.[3] We now wish to report work extending these initial experiments to esters and amides of other oxyacids of phosphorus using ammonia as reagent gas.

EXPERIMENTAL

Unless otherwise stated, all compounds were commercially available and were redistilled before use. Tetra-isopropyl esters of halogenated methylene bisphosphonates were prepared by the method of Quimby.[4] Diethyl phosphonoacetic acid was prepared by the method of Clayton *et al.*[5]

Mass spectra were obtained using an MS 80 mass spectrometer with a DS 55 data system (Kratos Analytical Instruments). CI mass spectra were recorded using ammonia as reagent gas at a pressure of 2×10^{-4} Torr in the source housing (equivalent to approximately 0.1 Torr in the source). Electron energy was 55 eV. The ion source temperature was 200 °C and the emission current 500 μA. EI mass spectra were obtained at 70 eV and a source temperature of 200 °C. Samples were introduced via a solids probe in the usual way.

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RESULTS AND DISCUSSION

Phosphonates

The CI spectra obtained for compounds **1–10** (Table 1) were similar to those obtained previously for phosphonates with hydrocarbons,[3] the major high molecular weight signal being due to the protonated molecular ions $[M+1]^+$. Useful structural data could be obtained from ammonia CI spectra. For example, the spectrum of tetra-isopropyl methylene bisphosphonate (**1**) (Fig. 1) contained peaks at *m/z* 345 (6.6%), 303 (4.0%), 261 (6.2%), 219 (3.2%) and 177 (3.5%), indicating the successive loss of C_3H_6 (42 u) and confirming the presence of a tetra-isopropyl ester (Table 2). The corresponding signals were scarely discernible in the EI spectrum of **1**. The CI spectra of the tetra-isopropyl esters of monochloro- (**2**), monobromo- (**3**) and dibromo- (**4**) -methylene bisphosphonates all showed similar families of peaks consisting of the protonated molecular ion and ions derived from the

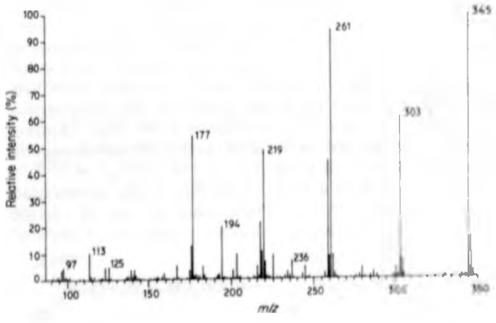

Figure 1. Ammonia CI mass spectrum of tetra-isopropyl methylene bisphosphonate (**1**).

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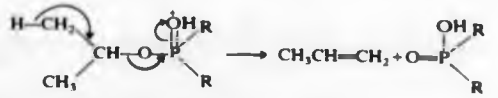
Table 1. Compounds studied

	Name	Formula	Elemental composition	Mol. wt
	Phosphonates			
1	Tetra-isopropyl methylene bisphosphonate	$(Me_2CHO)_2P(O)CH_2P(O)(OCHMe_2)_2$	$C_{13}H_{30}O_6P_2$	344
2	Tetra-isopropyl monochloromethylene bisphosphonate	$(Me_2CHO)_2P(O)CHClP(O)(OCHMe_2)_2$	$C_{13}H_{29}ClO_6P_2$	378 380
3	Tetra-isopropyl monobromomethylene bisphosphonate	$(Me_2CHO)_2P(O)CHBrP(O)(OCHMe_2)_2$	$C_{13}H_{29}BrO_6P_2$	422 424
4	Tetra-isopropyl dibromomethylene bisphosphonate	$(Me_2CHO)_2P(O)CBr_2P(O)(OCHMe_2)_2$	$C_{13}H_{28}Br_2O_6P_2$	500 502, 504
5	Triethyl phosphonoacetate	$(EtO)_2P(O)CH_2COOEt$	$C_8H_{17}O_5P$	224
6	Triethyl 2-phosphonopropionate	$(EtO)_2P(O)CHMeCOOEt$	$C_9H_{19}O_5P$	238
7	Diethyl phosphonoacetonitrile	$(EtO)_2P(O)CH_2CN$	$C_6H_{12}NO_3P$	177
8	Diethyl allylphosphonate	$(EtO)_2P(O)CH_2CH=CH_2$	$C_7H_{15}O_3P$	178
9	Benzyl diethyl phosphonoformate	$(EtO)_2P(O)COOCH_2Ph$	$C_{12}H_{17}O_5P$	272
10	Diethyl phosphonoacetic acid	$(EtO)_2P(O)CH_2COOH$	$C_6H_{13}O_5P$	196
	Phosphates			
11	Trimethyl phosphate	$(MeO)_3PO$	$C_3H_9O_4P$	140
12	Triethyl phosphate	$(EtO)_3PO$	$C_6H_{15}O_4P$	182
13	Tri-isopropyl phosphate	$(Me_2CHO)_3PO$	$C_9H_{21}O_4P$	224
14	Tri-*n*-butyl phosphate	$(BuO)_3PO$	$C_{12}H_{27}O_4P$	266
15	Triphenyl phosphate	$(PhO)_3PO$	$C_{18}H_{15}O_4P$	326
	Phosphites			
16	Trimethyl phosphite	$(MeO)_3P$	$C_3H_9O_3P$	124
17	Triethyl phosphite	$(EtO)_3P$	$C_6H_{15}O_3P$	166
18	Triphenyl phosphite	$(PhO)_3P$	$C_{18}H_{15}O_3P$	310
	Phosphoramidates			
19	Di-isopropyl phosphoramidate	$(Me_2CHO)_2P(O)NH_2$	$C_6H_{16}NO_3P$	181
20	Hexamethyl phosphoramide	$(Me_2N)_3PO$	$C_6H_{18}N_3OP$	179

successive loss of propene residues. This decomposition presumably arises from the protonation of the phosphoryl oxygen followed by elimination of olefin (Scheme 1). As might be expected, owing to the presence of halogen atoms, the protonated molecular ion and related ions in **2** were doublets (ratio 1 : 2.8), doublets (ratio 1 : 1) for **3** and triplets (ratio 1 : 2 : 1) for **4** (Table 2).

Structural information could also be obtained from the CI mass spectra of the ethyl esters **5**–**10**. In addition to the protonated molecular ion, other major ions corresponding to the successive loss of 28 u (presumably ethene) were present, confirming the presence of P—O—C_2H_5 residues. The CI mass spectra of **5** and **6** also showed ions corresponding to the loss of ethanol from the protonated molecular ion. This loss must involve the $COOC_2H_5$ group as this fragmentation does not occur in compounds **7**–**10**. The CI mass spectrum of the carboxylic acid diethyl phosphonoacetic acid (**10**) showed a peak due to $[M+NH_4]^+$ *m/z* 214 (4.2%), as well as a large peak due to the protonated molecular ion (29.5%). Peaks due to loss of carbon dioxide (*m/z* 153, 11.7%) and ethene (*m/z* 169, 1.9%) from the protonated molecular ion were also present.



Scheme 1. Decomposition of the protonated molecular ion of isopropyl ester of oxyacid of phosphorus.

Phosphates

Previous CI studies[3] did not include phosphate esters although their EI mass spectra have been studied extensively.[6] For phosphate esters, as might be expected, the major high molecular weight ion in their CI mass spectra was $[M+1]^+$. Again, families of peaks could be observed in the CI spectra from which information could be deduced as to the nature and number of groups bonded to phosphorus. Thus, the trialkyl esters **12**–**14** gave $[M+1]^+$ and ions due to the successive loss of three molecules of olefin (ethene, propene and butene respectively) (Fig. 2). Trimethyl (**11**) and triphenyl (**15**) phosphates showed little fragmentation in their CI mass spectra. The fragmentation of the trialkyl phosphates could be observed in their EI mass spectra. However, as the ion current carried by the molecular ion was less than 1% of the total ion current in all cases, the fragmentation patterns were much more difficult to observe than in the CI mass spectra.

Phosphites

Ammonia CI mass spectrometry was also useful for the detection of esters of oxyacids of trivalent phos-

Table 2. Principal ions and % total ion current carried in CI mass spectra of esters and amides of oxyacids of phosphorus

	Phosphonates
1	345 (6.6%), 303 (4.0), 261 (6.2), 219 (3.2), 177 (3.5)
2	381 (2.3%), 379 (6.6), 345 (3.2), 339 (1.9), 337 (5.3), 297 (1.2), 295 (3.6), 255 (0.6), 253 (1.7), 213 (0.3), 211 (1.0)
3	425 (1.3), 423 (1.3), 383 (1.3), 381 (1.3), 345 (2.9) 341 (0.8), 339 (0.8), 299 (0.3), 297 (0.3), 257 (0.3), 255 (0.3)
4	505 (1.7%), 503 (3.4), 501 (1.8), 463 (2.6), 461 (5.2), 459 (2.8), 421 (1.8), 419 (3.5), 417 (1.9), 379 (0.6), 377 (1.2), 375 (0.8)
5	225 (42.0%), 197 (9.5), 179 (5.3), 151 (3.9)
6	239 (29.6%), 211 (3.6), 193 (8.0), 183 (1.2)
7	195 (16.8%), 178 (52.0), 150 (3.4), 122 (1.1)
8	180 (11.3%), 179 (17.0), 178 (6.2), 167 (8.6), 151 (4.4), 134 (3.3), 123 (2.1)
9	273 (13.3%), 245 (0.3), 229 (12.6), 217 (0.2), 181 (8.8), 91 (29.3)
10	214 (4.2%), 197 (29.5), 183 (5.8), 169 (1.9), 153 (11.7)
	Phosphates
11	141 (23.3%), 110 (6.1), 94 (1.6), 79 (0.6)
12	183 (38.9%), 155 (20.0), 127 (5.4), 99 (0.6)
13	225 (22.8%), 183 (14.4), 141 (8.2), 99 (1.1)
14	267 (34.4%), 211 (14.8), 155 (8.0), 99 (1.6)
15	327 (40.3%), 233 (1.8), 170 (2.0)
	Phosphites
16	125 (10.8%), 111 (11.4), 94 (1.9)
17	167 (47.2%), 139 (20.9), 121 (2.5), 111 (2.2)
18	311 (10.4%), 235 (8.0), 217 (10.1), 153 (2.9), 94 (8.5)
	Phosphoramidates
19	182 (33.5%), 140 (14.6), 124 (3.9), 98 (14.9)
20	180 (22.3%), 136 (5.9), 135 (10.0), 92 (1.8)

phorus. In the three compounds tested (**16**–**18**), the protonated molecular ion carried at least 10% of the total ion current. For triethyl phosphite (**17**) peaks corresponding to the loss of two and not three molecules of ethene could be observed. There was little fragmentation observable in the CI mass spectrum of trimethyl phosphite (**16**), while the major fragmentation in the CI mass spectrum of triphenyl phosphite (**18**) corresponded to the loss of phenol (*m/z* 94) from the protonated molecular ion.

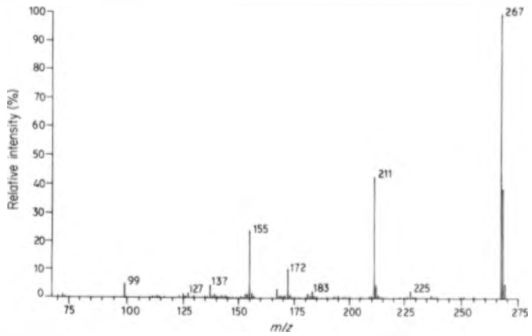


Figure 2. Ammonia CI mass spectrum of tri-*n*-butyl phosphate (**13**).

Phosphoramidates

The two phosphoramidates examined (**19** and **20**) showed strong peaks in their CI mass spectra due to protonated molecular ions. The major features in the CI mass spectrum of di-isopropyl phosphoramidate (**19**) were at *m/z* 140 (14.6%) and 98 (14.9%), corresponding to the successive loss of two molecules of propene. In this case, the protonated molecular ion carried 33.5% of the ion current carried by the sample, whereas in the EI mass spectrum the protonated molecular ion (the major, high molecular weight ion) carried only 0.3% of the total ion current. The CI mass spectrum of hexamethyl phosphoramide (**20**) showed only one major peak, in addition to $[M+H]^+$ (22.3%), corresponding to the loss of 45 u, presumably Me_2NH.

Acknowledgements

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ORGANOPHOSPHORUS COMPOUNDS AS ANTIVIRAL AGENTS

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(*Received September 24, 1982*)

The 5'-triphosphates of 2'-5' linked oligoadenylic acids are formed in cells which have been exposed to interferon and may be involved in the antiviral activity of the latter. The lead(II) ion-catalysed oligomerisation of adenosine 5'-phosphorimidazolidate is a convenient route for the preparation of the 5'-phosphates of 2'-5' linked oligoadenylic acids. The latter can readily be converted to the triphosphates or coupled to the 5'-phosphate of nicotinamide nucleoside to give naturally occurring pyrophosphates which may act as reservoirs for the oligoadenylic acids in cells.

Pyrophosphate analogues, eg, phosphonoacetic and phosphonoformic acids or carbon-substituted methylenebisphosphonic acids are antiviral agents of potential commercial interest as they inhibit the replication of a number of viruses including herpes and influenza. These pyrophosphate analogues do not appear to inhibit virus replication by being incorporated into nucleoside triphosphates which block nucleic acid synthesis. Rather the analogues appear to act by forming stable complexes with an essential metal ion (probably zinc) at the active sites of nucleic acid polymerases of viruses.

At the present time, most bacterial infections can be controlled by chemotherapeutic agents as many bacterial enzymes are unlike those of the host organism. On the other hand, the enzymes of viruses are often very similar to those of the host organism and hence the chemotherapeutic control of viruses is difficult. There are, however, some small differences between host and viral enzymes and these have been exploited recently in a number of laboratories. Particular progress in the chemotherapy of virus infections has been made in three areas: (a) nucleoside analogues, (b) interferon, and (c) low molecular weight inhibitors of viral enzymes. Nucleoside analogues are outside the scope of this review but I would like to consider briefly some progress which is being made in the interferon field before concentrating on the low molecular weight inhibitors.

Interferon is a naturally occurring glycoprotein which can confer antiviral properties on cells. When interferon acts on susceptible cells, the synthesis of some unusual oligonucleotides, eg, the 5'-triphosphate of adenylyl(2'-5')adenylyl(2'-5')adenosine (1) and higher oligomers is enhanced. This oligonucleotide is unusual as it is the only naturally occurring nucleic acid derivative to be discovered at the present time which contains 2'-5' rather than the more normal 3'-5' internucleotide links. These oligomers have a powerful inhibitory effect on protein synthesis in cells even when they are present at very low concentrations. One important sequence of events which is triggered within the cell by (1) is the phosphorylation of a nuclease and this can then

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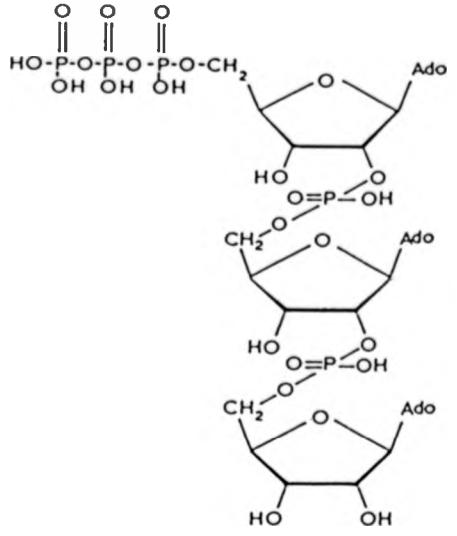


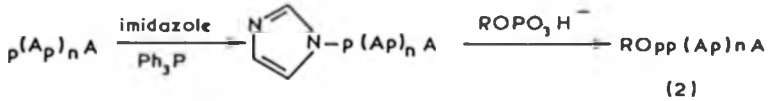
(1)

degrade single stranded RNA. As the mRNA of viruses is single stranded the activated nuclease prevents viral replication.

Thus, the oligonucleotide (1) would seem to have potential as an antiviral agent. However, at least three problems have to be overcome before (1) can be used. These are (a) the synthesis of substantial amounts of the oligonucleotides, (b) the poor uptake of (1) by the cells and (c) their low stability in cells. There are some twenty syntheses of (1) in the literature[1] but most of these are complex, multistage procedures involving protecting groups and the overall yields are low. An attractive method for the synthesis of (1) is based on prebiotic models for oligonucleotide synthesis.[2] Oligomerisation of the 5'-phosphorimidazolidate of adenosine in the presence of lead(II) ions gives the 5'-phosphates of predominantly 2'-5' linked oligoadenylic acids. Some 3'-5' linked oligomers are also formed but these are easy to remove by enzymic hydrolysis. No protection of the sugar hydroxyls or the adenine residue are required and reasonable yields of the 5'-phosphates of the 2'-5' linked oligoadenylic acids can be obtained in only a few days. The latter can then be converted into the corresponding 5'-triphosphates via their phosphoromorpholidates. The oligoadenylic acids are highly charged and are not taken up readily by cells. Various methods have been used to overcome this problem, the most successful appears to be precipitation as the calcium salts.[3] This is feasible as a research technique but obviously presents problems for *in vivo* work. Even though the oligoadenylic acids related to (1) seem to be unique in possessing 2'-5' internucleo-

tide links and hence must have a very different shape to the 3'-5' linked isomers, there are enzymes in cells which recognise these compounds and degrade them in minutes. One way to stabilise (1) is to block the 2',3'-hydroxyl groups by alkylation and this aspect is under investigation in a number of laboratories. We have been intrigued by the isolation of a nicotinamide derivative (2) of the oligoadenylic acid trimer.[4] The biological function of (2) is not known but it has been suggested that it may act as a reservoir for (1) which can be formed by hydrolysis and further phosphorylation. As (2) only occurs in minute amounts in cells we have devised a synthesis based on the coupling of nicotinamide ribonucleoside 5'-phosphate with the 5'-phosphorimidazolidates of 2'-5' linked oligoadenylic acids prepared by the lead(II) ion-catalysed method.

The biological properties of (2) are being investigated at the Imperial Cancer Research Foundation in London.



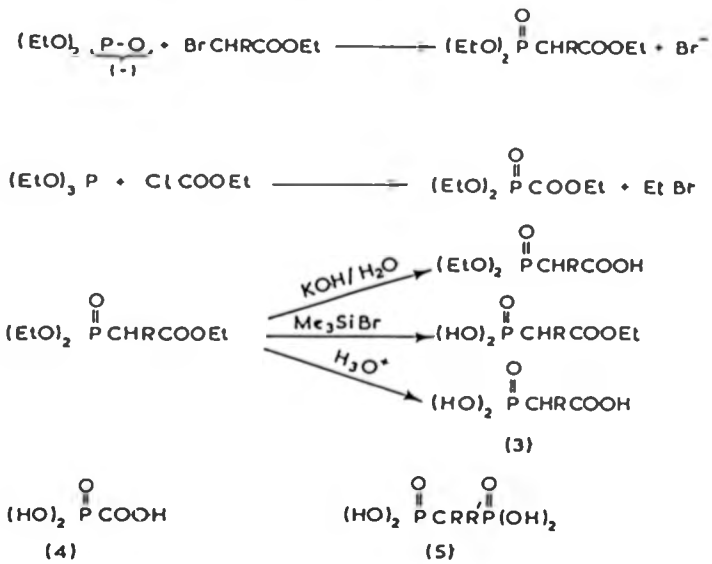
(2)

where R = nicotinamide –1–(3–β–D–ribofuranosyl) 5'

Although oligoadenylic acids related to (1) have antiviral properties under suitable conditions, problems outlined above associated with production, uptake and stability do not make them promising antiviral agents for clinical use at the moment. Low molecular weight inhibitors of viruses are more attractive commercially as they should be simple compounds and hence comparatively easy to manufacture. Moreover, it should be possible to introduce variations in structure by synthetic methods to overcome problems of uptake, toxicity, stability, etc., without undue difficulty.

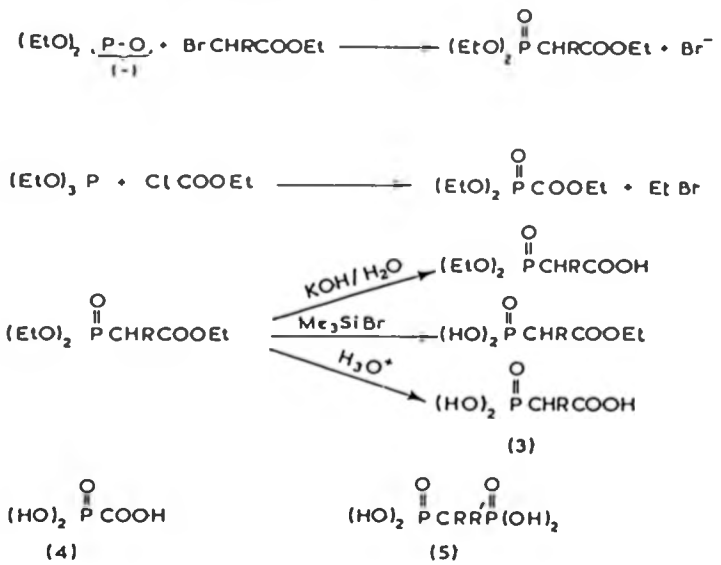
One promising class of low molecular weight inhibitors of virus replication which we have been studying are analogues of pyrophosphoric acid. Examples of these analogues, phosphonoacetic (3, R = H) and phosphonoformic (4) acids have been known for many years and they can be prepared either by the Michaelis Becker[5] or Arbusov reactions.[6] Since (3) and (4) contain both carboxylic and phosphoric acid residues it is a simple matter to prepare derivatives modified at either residue.[7,8] Another class of pyrophosphate analogues which we have found to be effective antiviral agents are carbon-substituted methylenebisphosphonic acids (5).

We have prepared a number of derivatives of (3–5) and required a simple way to analyse our products. Nuclear magnetic resonance spectroscopy was not very useful and hence we turned our attention to mass spectrometry. Electron impact mass spectrometry was unsatisfactory as the parent molecule could not easily be detected as it carried only a very small percentage of the total ion current. Chemical ionisation mass spectrometry using ammonia as the reagent gas gave spectra in which the protonated molecular ion carried 10–30% of the total ion current.[9] Not only were intense molecular ions present but useful structural information could be obtained as only limited fragmentation of the protonated molecular ions occurs with



ammonia as reagent gas. In the ammonia chemical ionisation mass spectrum of triethyl phosphonoacetate, the $(M + H)^+$ peak appears at m/z 225 and there are peaks corresponding to the successive loss of ethylene residues (28 mass units) at m/z 197, 169 and 141 confirming the presence of a triethyl ester. The successive dealkylation of phosphate esters during ammonia chemical ionisation mass spectrometry is quite general and for example, tetraisopropyl methylenebisphosphonate shows a $(M + H)^+$ peak at m/z 345 and peaks at m/z 303, 261, 219 and 177 corresponding to the successive loss of four propylene residues (42 mass units) (Figure 1). In this manner we have been able to analyse halogenated and other derivatives of (3–5).

The pyrophosphate analogues (3–5) are effective inhibitors of virus replication (Table 1).[10,11] They act by inhibiting nucleic acid synthesis and the presence of the free carboxylic acid and both phosphoryl hydroxyl groups in (3) and (4) or all phosphoryl hydroxyl functions in (5) is essential for antiviral activity. Modification of any of the acid functions in these compounds causes almost complete loss of antiviral activity. The initial discovery of the antiviral properties of (3) and (4) was carried out with herpes virus[12] but influenza virus, cytomegalovirus, and a number of other viruses, are also inhibited. Nucleic acid synthesis is inhibited by inhibition of the nucleic acid polymerases in the viruses but some nonviral polymerases are also sensitive such as DNA polymerase α of mammalian cells,[12] and reverse transcriptase (the RNA dependent DNA polymerase of certain tumour viruses).[13]



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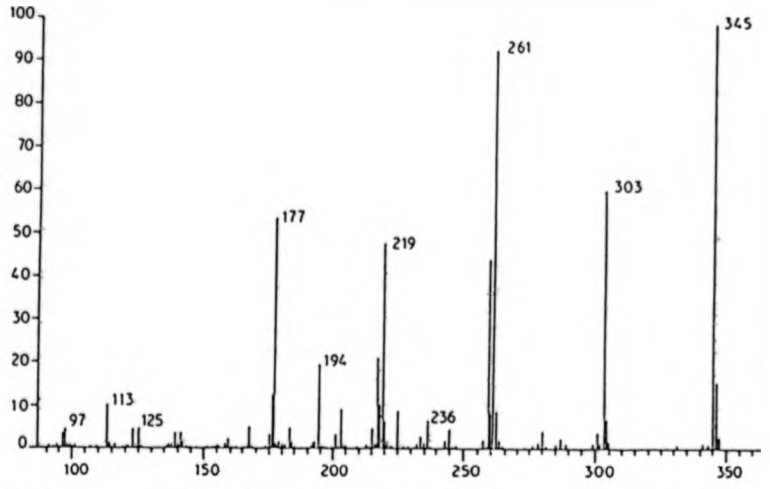


FIGURE 1 Ammonia chemical ionisation mass spectrum of tetraisopropyl methylenebisphosphonate.

We have been studying the effect of pyrophosphate analogues on influenza virus mainly because of availability and ease of handling of the virus. However, there are problems as it is difficult to isolate from influenza virus active RNA polymerase which is free from other proteins. The native nucleic acid template must be present together with an oligonucleotide promoter which makes interpretation of the enzyme kinetics of the polymerase reaction difficult. The inhibition of influenza RNA

TABLE I

Inhibitory effect of pyrophosphate analogues on RNA polymerase from influenza virus

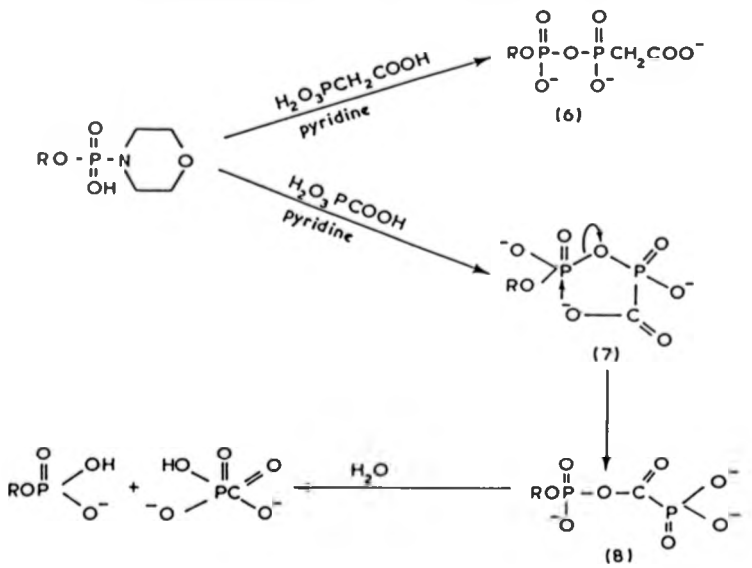
Compound	Concentration (μM) Producing 50% Inhibition of Polymerase
$(HO)_2P(O)CH_2COOH$	275
$(HO)_2P(O)COOH$	35
$(HO)_2P(O)CH_2CH_2COOH$	> 500
$(HO)_2P(O)CH_2CONH_2$	> 500
$(HO)_2P(O)OP(O)(OH)_2$	125
$(HO)_2P(O)NHP(O)(OH)_2$	50
$(HO)_2P(O)CH_2P(O)(OH)_2$	> 500
$(HO)_2P(O)CHClP(O)(OH)_2$	85
$(HO)_2P(O)CCl_2P(O)(OH)_2$	75
$(HO)_2P(O)CBr_2P(O)(OH)_2$	10
$(HO)_2P(O)COP(O)(OH)_2$	20

polymerase is followed by studying the effect of pyrophosphate analogues on the incorporation of radioactivity into acid-insoluble polynucleotide. This is more rapid and a much more sensitive assay than ones based on inhibition of viral growth.

There are two plausible mechanisms for the mode of action of the pyrophosphate analogues. Either, (a) they are converted by host or virus into analogues of nucleoside triphosphates which inhibit the viral polymerases, or (b) they interact directly with the polymerases possibly by coordinating with an essential metal ion. We do not believe that these inhibitors act by being incorporated into the β-γ positions of a nucleoside triphosphate analogue. We have prepared the ATP analogue of phosphonoacetic acid (6, R = adenosine-5') and this compound is neither an inhibitor of nor a substrate for RNA polymerase from influenza virus. The similar thymidine analogue (6, R = thymidine-5') is neither an inhibitor of nor a substrate for DNA polymerase from herpes virus.[16] Dichloromethylenebisphosphonic acid (5, R = R^1 = Cl) is a good inhibitor of influenza virus RNA polymerase but again its ATP analogue is neither an inhibitor of nor a substrate for the polymerase.

The ATP analogue (6, R = adenosine-5') is easy to prepare by the phosphoromorpholidate route but when we tried to prepare the ATP analogue of phosphonoformic acid all we achieved was the rapid conversion of adenosine 5'-phosphoromorpholidate into AMP. The reaction in pyridine can be followed by ^{31}P NMR at 162 MHz using a Bruker WH400 spectrometer. With phosphonoacetic acid there was a steady decay of the phosphoromorpholidate signal at 7.8 ppm matched by an increase in intensity of a pair of doublets centred at 5.2 and -9.7 ppm due to the formation of the pyrophosphate residue in (6, R = adenosine-5'). With phosphonoformic acid, the phosphoromorpholidate signal decays as before but no new doublets can be detected, only a new singlet at 2.2 ppm due to adenosine 5'-phosphate. If the C-ethyl ester of phosphonoformic acid is used in place of the free acid, signals at 28.2 ppm due to the ester and 7.8 due to the adenosine 5'-phosphoromorpholidate can be observed at the start of the experiment. With time, two doublets centred at 20.2 and -9.4 ppm appear due to the formation of the pyrophosphate. Thus the C-ethyl ester of phosphonoformic acid which is not an inhibitor of the viral polymerase does form a nucleoside triphosphate analogue. We believe that phosphonoformic acid reacts with adenosine 5'-phosphoromorpholidate to give an ATP analogue (7) but this breaks down very rapidly in an intramolecular reaction to give a mixed anhydride of adenosine 5'-phosphoric and formic acids (8). Such mixed anhydrides are considerably more reactive than phosphoric-acetic mixed anhydrides[17] and would be expected to decompose rapidly in pyridine. With phosphonoacetic acid, the nucleoside triphosphate analogue could still decompose by an intramolecular reaction but here a six-membered rather than a five-membered ring would be involved and hence this breakdown should be comparatively slow.

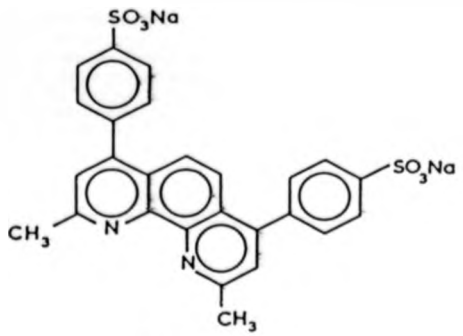
Marked differences between the rates of intramolecular hydrolysis of phosphonate esters have been observed depending on the size of the ring involved in the cyclic reaction. Thus, diethyl 2-carboxymethylphenylphosphonate undergoes intramolecular hydrolysis 10^5 times more slowly than diethyl 2-carboxyphenylphosphonate at pH 3.0 and 79.5°.[18] The C-ethyl ester of phosphonoformic acid cannot decompose via a cyclic reaction as the carboxyl group is blocked. Hence, the formation of the pyrophosphate bond in the nucleoside triphosphate analogue can be observed.



Many DNA and RNA polymerases are zinc-requiring enzymes[19,20] and we propose that phosphonoacetic, phosphonoformic and methylenebisphosphonic acids act by complexing with this zinc ion preventing the release of pyrophosphate in the chain-elongation step of the polymerisation. This proposal is not new[21] but so far little evidence has been assembled to support it. A study of enzyme kinetics using DNA polymerase from herpes virus shows that both phosphonoacetate and pyrophosphate inhibit the enzyme in an analogous manner but that pyrophosphate is the less active. Both are non-competitive inhibitors of deoxyribonucleoside triphosphates in the polymerase reaction. In addition, phosphonoacetate is a competitive inhibitor of pyrophosphate in the pyrophosphate ⇄ deoxyribonucleoside triphosphate exchange reaction which is catalysed by the polymerase.[22] This evidence suggests that phosphonoacetate blocks a site on the enzyme which binds pyrophosphate. Further support for the chelation mechanism of inhibition is the observation that bathocuproin (9) and 2-acetylpyridine thiosemicarbazone (10), both good chelating agents for "soft" metal ions such as zinc, inhibit influenza virus replication by inhibiting the RNA polymerase of the virus.[23]

Little has been published on the metal-chelating properties of pyrophosphate analogues but one study[24] does show that phosphonoacetate chelates zinc strongly.

We are investigating the metal chelating properties of our antiviral compounds and hope to see a correlation between the stability constants of the zinc complexes with antiviral activity.



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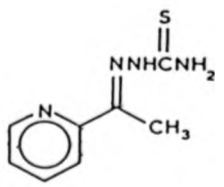

(10)

Thus, from in vitro studies, pyrophosphate analogues appear to have promise as chemotherapeutic agents. Those simple derivatives tested so far appear to suffer from one drawback. Since they are good chelators of divalent metal ions they also complex strongly with calcium ions and are deposited in the bone. The methylene-bisphosphonate (5, R = R' = Cl) has been proposed for the treatment of Paget's disease of the bones. Pharmaceutical companies are naturally wary of marketing an antiviral which may affect the composition of bones, although there has been a suggestion that phosphonoformic acid may find use as an anti-herpes ointment for topical application. Our research into the mode of action of pyrophosphate analogues may lead to the development of other, active compounds which do not have unfortunate side effects.

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The inhibition of the RNA polymerase activity of influenza virus A by pyrophosphate analogues

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ABSTRACT

Substituted methylene diphosphonates are effective inhibitors of the RNA polymerase of influenza A virus causing 50% inhibition of the polymerase activity when they are present in the concentration range 10-85 μM. The inhibitory power of the methylene diphosphonates appears to be related to their ability to chelate with metal ions.

INTRODUCTION

Influenza A virus has associated with its core an RNA-dependent RNA polymerase activity which differs from the polymerases of host cells[1]. Any differences in properties between the two classes of polymerase activity might be useful for chemotherapeutic exploitation. Pyrophosphate analogues (eg, phosphonoacetate (1) or phosphonoformate (2)) have been shown to inhibit the RNA polymerase of influenza virus[3]. We now wish to report that substituted methylene diphosphonates, another class of pyrophosphate analogues, inhibit this RNA polymerase activity under conditions when DNA polymerase α is little affected.

MATERIALS AND METHODS

Reagents

Adenylyl(3'-5')guanosine and trisodium phosphonoformate (2) (Sigma Chemical Co), imidodiphosphonate (8) (Boehringer Corporation) and 3-phosphopropionic acid (3) (Fluka AG) were available commercially. [5-^{3}H]UTP (42 Ci/mmol) and ^3H_2O (100 Ci/mol) were obtained from Amersham International. Phosphonoacetic (1) and 2-phosphonopropionic (4) acids were prepared by hydrolysis of their triethyl esters[4]. Diethyl phosphonoacetic acid (6) was

prepared by the method of Clayton *et al*[5]. Methylene diphosphonic (9)[6] and substituted methylene diphosphonic acids (10-13)[7] were synthesised as described. The C-amide of phosphonoacetic acid (5) was prepared by the action of ammonia on the corresponding ethyl ester.

Virus Type and Preparation

The influenza virus A/X49 was a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. The virus was grown in the allantoic sac of fertile hens' eggs and was isolated essentially as described[8]. Eleven day old embryonated hens eggs were inoculated with infected allantoic fluid (0.1 ml of a 10^{-3} dilution in phosphate buffered saline), the eggs were incubated (33°C/48 hrs) and then chilled (-20°C/2 hrs). The allantoic fluid was collected, and centrifuged (3000 rpm/20 min) to remove unwanted egg membranes, from this point onwards all procedures were carried out at 0 to 4°C. The supernatant was removed and the virus was pelleted by centrifugation (21,000 rpm/ 90 min, 6 x 250 ml rotor). The supernatant was discarded and the virus pellet was allowed to soak overnight in PBS. The pellet was then resuspended in PBS and layered on to a velocity gradient of 10 to 40% (w/v) sucrose in buffer (30 ml, 10 mM tris-HCl, pH 7.4) and centrifuged (22,000 rpm/1 hr, 3 x 65 ml swing-out rotor). The diffuse virus band was collected by bottom puncture and the sucrose was diluted out with PBS to a final volume of 30 ml. The virus suspension was layered on to an equilibrium gradient of 30 to 70% (w/v) sucrose in buffer (30 ml, 10 mM tris-HCl, pH 7.4) and centrifuged (20,000 rpm/overnight, 3 x 65 ml swing out rotor). The virus band was collected, diluted with PBS and the virus pelleted by centrifugation (30,000 rpm/2 hrs, 8 x 50 ml rotor). The supernatant was discarded and the pellet was allowed to soak overnight in PBS. The virus was then resuspended in buffer (3 ml, 400 µM tris-HCl, pH 8.0) and frozen as aliquots at -70°C and thawed once prior to use.

RNA Polymerase Assay

The standard reaction mixture contained in 200µl:50 mM *tris*-HCl, pH 8.0; 5 mM magnesium acetate; 150 mM potassium chloride; 5 mM dithiothreitol; 0.4 mM ApG; 0.25% (v/v) Nonidet P-40; 0.4 mM

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action mixture contained in 200µl:50 mM *tris*-
gnesium acetate; 150 mM potassium chloride;
0.4 mM ApG; 0.25% (v/v) Nonidet P-40; 0.4 mM

each of ATP, CTP, GTP and [^{3}H] UTP (5 µCi) and purified virus (10 µl, 2000 Hau). Mixtures were kept at 4°C until zero time of reaction, polymerisation being initiated by addition of virus. The mixture was kept at 30°C for 1 hour during which time the incorporation of tritium into acid-precipitable material increased in a linear fashion. After 1 hour, cold saturated sodium pyrophosphate solution (200 µl) followed by cold TCA (2 ml, 10% w/v) were added and the mixture kept on ice for 15 minutes after thorough agitation. Precipitated material was collected on Whatman GF/C discs which were washed several times with 10% TCA, once with ethanol and dried. The radioactivity of material precipitated on the discs was then determined by scintillation counting using a toluene-based medium. Pyrophosphate analogues were added to the reaction mixtures before addition of virus. The concentration of analogue which inhibited by 50% the incorporation of [^{3}H]uridine into acid insoluble material was derived from the dose-response curve for each compound.

DNA Polymerase α Assay

The enzyme from calf thymus was obtained from Worthington and was assayed as described by Matsukage *et al*[9].

[^{3}H]-Labelled Phosphonoacetic Acid (1)

To anhydrous phosphonoacetic acid (1) (5 mg) dissolved in carrier free ^3H_2O (25 µl) was added concentrated HCl (1 µl) and the mixture heated at 70°C for 5 hours. Excess ^3H_2O was removed under pressure and exchangeable tritium removed by repeated addition and evaporation of water. The residue, which co-chromatographed on paper with unlabelled (1) in EtOH:1 M ammonium acetate (5:2 v/v), was dried *in vacuo* over P_2O_5 to give [2-^{3}H]-phosphonoacetic acid, specific activity 12 µCi/µmole).

Recovery of [2-^{3}H]-Phosphonoacetate (1) from RNA Polymerase Assay

To the standard assay solution (1 ml), containing 0.4 mM UTP in place of [^{3}H]-UTP, was added [2-^{3}H]-(1) (0.5 µmole) and purified virus. The reaction was incubated at 30°C for 1 hour then EDTA was added to a final concentration of 20 mM. The reaction mixture was applied to a DEAE DE-52 column (OAc^- form, 0.6 x 14 cm) which was washed with water and then eluted with a linear gradient of triethylammonium acetate (0.5µM, pH 4.7, 100 ml). The radioactivity of the fractions (1.5 ml) was then determined by

liquid scintillation counting, the recovery of radioactivity from this experiment was almost 90%. The relative positions of elution of [^{3}H]-(1) and adenyl phosphonoacetate (AMPPAA)[10] were determined in a separate experiment.

Determination of Pyrophosphate Analogue - Zinc Ion Stability Constants

The stability constants were determined by the method of Hummel and Dreyer[11], using a column of Sephadex G-10 (1.6 x 92 cm) which had been equilibrated with zinc chloride (10 μM) in triethanolamine-HCl buffer (0.1 M, pH 8.0). The analogues (100 nmole) were added in triethanolamine/zinc buffer (1 ml) and the column eluted with the same buffer at a flow rate of 0.4 ml/min. The zinc content of individual fractions (2 ml) was determined by atomic absorption spectrometry and the stability constants determined as described[11].

RESULTS AND DISCUSSION

Substituted methylene diphosphonates (10-13) are effective inhibitors of the RNA polymerase activity of influenza virus A (Table), causing 50% inhibition of the polymerase at concentrations at which mammalian DNA polymerases (represented by calf thymus DNA polymerase α) are not affected. It has been reported that both (1) and (2) inhibit influenza RNA polymerase[3] and we have observed that both these compounds are potent inhibitors of DNA polymerase α. Compounds (1) and (2) are effective antiviral agents against Herpes viruses and it has been suggested[12] that they are active by virtue of their ability to chelate with an essential metal ion in the Herpes DNA polymerase and hence inhibit DNA synthesis. We propose that the methylene diphosphonates (10-13) act in a similar manner against influenza virus A. The RNA polymerase activity of influenza viruses is zinc-requiring[13] and can be inhibited by compounds, eg, 2-acetylpyridine thiosemicarbazone, which can chelate with 'soft' heavy metal ions. The pyrophosphate analogues (10-13) do not appear to exert their inhibitory effect by removing from solutions magnesium ions which are essential for the RNA polymerase as magnesium ions are present in the polymerisation reaction at a concentration of 5 mM while the analogues are effective inhibitors of the polymerase at concentrations around 50 μM. The pyrophosphate analogues do not appear

TABLE

INHIBITORY EFFECT OF PYROPHOSPHATE ANALOGUES ON RNA POLYMERASE FROM INFLUENZA VIRUS AND CALF THYMUS DNA POLYMERASE α

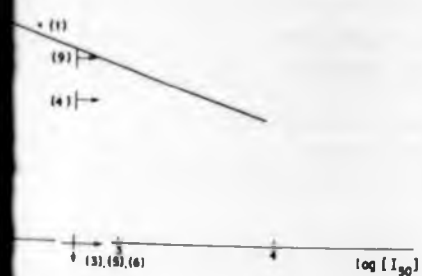
	Compound	pK_d,*	Concn. (μM) producing 50% inhibition	
			'flu RNA polymerase	DNA polymerase α
(1)	RCH_2COOH	5.5	275	45
(2)	RCOOH	5.6	35	35
(3)	RCH_2CH_2COOH	< 4	> 500	> 500
(4)	RCHMeCOOH	∿ 5	> 500	> 500
(5)	$RCONH_2$	< 4	> 500	> 500
(6)	$(EtO)_2P(O)CH_2COOH$	< 4	> 500	> 500
(7)	ROR	5.7	125	> 500
(8)	RNHR	5.7	50	> 500
(9)	RCH_2R	5.3	> 500	> 500
(10)	RCHClR	> 6	85	> 500
(11)	$RCCl_2R$	> 6	75	> 500
(12)	$RCBr_2R$	> 6	10	350
(13)	RCOR	5.4	20	100

where R = $(HO)_2P(O)$

*Dissociation constant of complex formed with zinc ions, measured at pH 8.0 as described in text.

to inhibit the RNA polymerase activity of influenza by being converted into analogues of nucleoside triphosphates before acting on the enzyme. Adenyl phosphonoacetate[10] is neither a substrate for nor an inhibitor of the polymerase activity. Furthermore, when [2-^{3}H]-phosphonoacetate was incubated with the standard polymerase assay mixture, all the radioactivity receoverd from the assay was in the form of starting material, none could be detected in the form of nucleoside triphosphate analogues.

Little data on the metal chelating properties of pyrophosphate analogues have been published. We have, therefore, determined by gel filtration a dissociation constant K_d, (Table) for



)[μM] producing 50% inhibition.

(the ability of pyrophosphate
pH 8) and their effectiveness as
se activity of influenza A virus.
, PFA phosphonoformate (2), 3PPA 3-
2-phosphonopropionate (4), PP_i
hloromethylene diphosphonate (10),
sphonate (11), Br_2MDP dibromomethy-
arbonyl diphosphonate (13).

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NA polymerase assays are carried
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compounds (1) [5.3][14], and (11)
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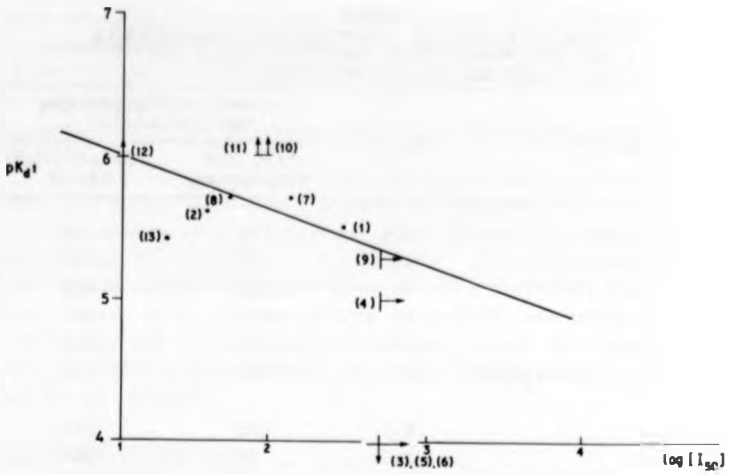
za (Figure). Thus, (1) and (2)

have relatively high $pK_{d's}$ and are inhibitors of the polymerase, whereas (3)-(6) have lower $pK_{d's}$ and are ineffective inhibitors. Steric factors may also be important and (2) which can form a metal chelate with a 5-membered ring is a more effective inhibitor than (1) which forms a chelate with a 6-membered ring. There is a correlation between $pK_{d'}$ and polymerase inhibitors for methylene diphosphonates, and (9) which has a lower $pK_{d'}$ than the halogenated analogues (10)-(12) is an ineffective inhibitor of the polymerase. On the other hand the analogues (10)-(12) are good inhibitors. Interestingly, carbonyl diphosphonate (13) is a more effective inhibitor of the RNA polymerase activity than (9) though it has a similar $pK_{d'}$. Here, again, steric factors may be important as the P-C-P angle in the two compounds should be very different. Inorganic pyrophosphate (7) and imidodiphosphonate (8) have high $pK_{d's}$ and are inhibitors of the polymerase. Presumably the inhibition of the polymerase activity by inorganic pyrophosphate is due to suppression of the dissociation of the enzyme-inorganic pyrophosphate complex which is present after the formation of the internucleotide bond. The kinetics of the inhibition of influenza virus RNA polymerase activity by the pyrophosphate analogues are difficult to follow owing to the complexity of the enzyme system (for example, Lineweaver-Burk plots of the inhibition of this enzyme activity by pyrophosphate analogues are curved). Furthermore, the polymerase activity is inhibited by excess zinc, making it difficult to determine whether the inhibition of the enzyme activity by the pyrophosphate analogues can be reversed by the addition of an excess of zinc ions.

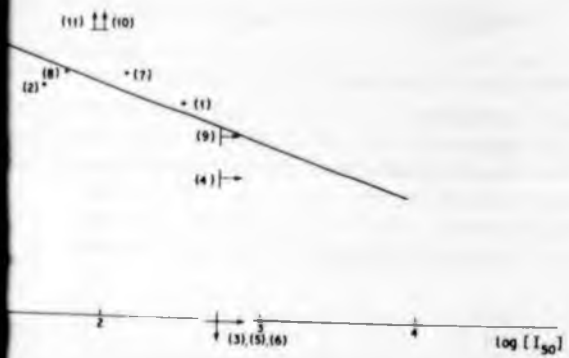
We suggest that the evidence presented above supports the hypothesis that pyrophosphate analogues inhibit the RNA polymerase activity of influenza virus by complexing with an essential zinc ion at the active site of the enzyme and preventing the binding of nucleoside triphosphates or preventing the release of inorganic pyrophosphate once the internucleotide bond has been formed by the enzyme. One reason for the difference in sensitivity of different polymerases (eg, DNA polymerase α) to these analogues may be that the environment around the essential zinc ion is such that an effective inhibitory complex cannot be formed in the insensitive enzymes. Investigations on other strains of



I_{50} = Concentration [μM] producing 50% inhibition

FIGURE
Relationship between K_d, (the ability of pyrophosphate analogues to bind zinc ion at pH 8) and their effectiveness as inhibitors of the RNA polymerase activity of influenza A virus.
PAA phosphonoacetate (1), PFA phosphonoformate (2), 3PPA 3-phosphonopropionate (3), 2PPA 2-phosphonopropionate (4), PP_i pyrophosphate (9), ClMDP monochloromethylene diphosphonate (10), Cl_2MDP dichloromethylene diphosphonate (11), Br_2MDP dibromomethylene diphosphonate (12), CDP carbonyl diphosphonate (13).

complexes formed between zinc ions and pyrophosphate analogues at pH 8.0, the pH at which the RNA polymerase assays are carried out. We do not attempt to specify which ligand species are involved in complex formation as more than one species may be present and K_d, is merely an indication of the strength of the interaction between the analogues and zinc ions at this pH. However, our pK_d, values do not differ markedly for the pK_d values reported for the fully ionised compounds (1) [5.3][14], and (11) [6.7][15]. We find that there is a correlation between the pK_d, of an analogue and its effectiveness as an inhibitor of the RNA polymerase activity of influenza (Figure). Thus, (1) and (2)



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